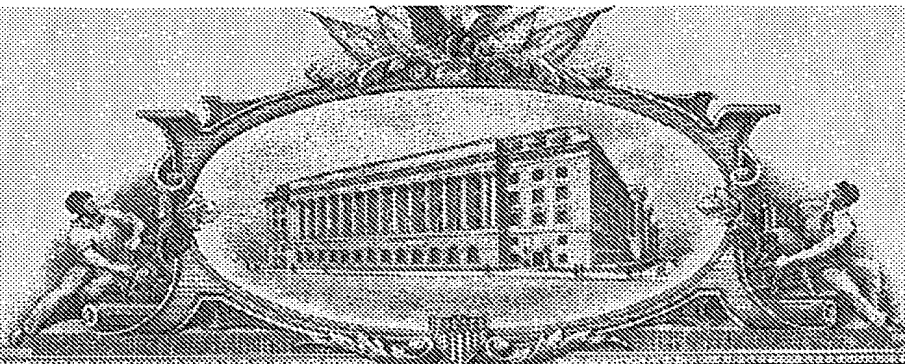


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**APPLICATION NUMBER: 60/508,034  
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### PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53 (c).

Express Mail Label No. EV 313 980 888 US

INVENTOR(S)				
Given Name (first and middle [if any])		Family Name or Surname		Residence (City and either State or Foreign Country)
Sharat		SINGH		San Jose, California, U.S.A.
<input type="checkbox"/> Additional inventors are being named on the _____ separately numbered sheets attached hereto				
TITLE OF THE INVENTION (500 characters max)				
DETERMINING APOPTOTIC STATUS IN NEOPLASTIC DISODERS				
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<input checked="" type="checkbox"/> Customer Number <u>33603</u>				
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ENCLOSED APPLICATION PARTS (check all that apply)				
<input checked="" type="checkbox"/> Specification Number of Pages <u>39</u> <input type="checkbox"/> CD(s), Number _____				
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<input type="checkbox"/> Application Data Sheet. See 37 CFR 1.76				
METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT				
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<input checked="" type="checkbox"/> The Director is hereby authorized to charge filing fees or credit any overpayment to Deposit Account Number: <u>50-2266</u>				
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FILING FEE AMOUNT (\$) <u>\$80.00</u>				
The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.				
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(Page 1 of 1)

Respectfully submitted

SIGNATURE

TYPED or PRINTED NAME

Stephen C. Macevitz

TELEPHONE

(650) 210-1223

Date: 01 October 2003

Registration No.: 30,285

Docket No.: 134.00USP

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(Print Name of Person Mailing Application)

  
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Case No. 134.00USP

**DETERMINING APOPTOTIC STATUS IN NEOPLASTIC DISORDERS**

5

**Field of the Invention**

The present invention relates generally to methods for determining apoptotic status, and more particularly, to methods for measuring molecular complexes that form during the up-regulation or down-regulation of one or more apoptotic pathways.

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**Background of the Invention**

The formation and disassociation of molecular complexes are crucial to regulatory processes in living organisms. In particular, signaling pathways between the extracellular environment and the nucleus of a cell involve the formation of many molecular complexes in which multiple proteins are assembled to directly or indirectly induce molecular events, such as phosphorylation or dephosphorylation, which are steps in the signaling process, Gomperts et al, Signal Transduction (Academic Press, New York, 2002). Such pathways and their components have been the subject of intense investigation because of the role aberrant pathway behavior plays in many disease conditions, especially cancer, e.g. McCormick, Trends in Cell Biology, 9: 53-56 (1999); Blume-Jensen and Hunter, Nature, 411: 355-365 (2001); Nicholson et al, Cellular Signalling, 14: 381-395 (2002); and the like. It has been observed that many cancers are associated with an accumulation of mutations or other genetic alterations that affect components of signaling pathways, e.g. by over expression, particularly those pathways involved with cell proliferation, cell motility, differentiation, and cell death, e.g. Blume-Jensen and Hunter (cited above).

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In particular, many cancers appear to be caused by malfunctions in one or more signal transduction pathways that are involved in the regulation of programmed cell death, or apoptosis, e.g. Evan and Vousden, *Nature*, 411: 342-348 (2001). Such observations have led to new approaches for treating a variety of cancers based on the inhibition of a single protein in a single pathway, for example, by preventing complex formation or by inhibiting a kinase activity, Korsmeyer, U.S. patent 5,834,209; Baltimore et al, U.S. patent 6,410,516; Vivanco et al, *Nature Reviews Cancer*, 2: 489-501 (2002); and the like. Unfortunately, the practical development of such approaches has been difficult because of the redundancy and inter-connectedness of signaling pathways. Not only do there appear to be multiple pathways mediating programmed cell death, but also components a pathway are often activated or de-activated by multiple routes, e.g. involving different kinases, binding proteins, or the like (for example, Gilmore et al, *J. Biol. Chem.*, 277: 27643-27650 (2002)). A key apoptotic pathway, sometime referred to as the “mitochondrial apoptotic pathway,” involves the Bcl-2 family of proteins. Members of this family interact with each other and with non-member cellular components to determine whether cell death or cell survival is promoted, e.g. Aontonsson, *Cell Tissue Res.*, 306: 347-361 (2001); Gross et al, *Genes & Development*, 13: 1899-1911 (1999); Otilie et al, *J. Biol. Chem.*, 272: 30866-30872 (1997); Franke et al, *Nature*, 390: 116-117 (1997); Sattler et al, *Science*, 275: 983-985 (1997). Cell death follows from events induced in mitochondria, such as an increase in membrane permeability and release of cytochrome c, which, in turn, activate proteolytic enzymes, such as caspases. Within the mitochondrial apoptotic pathway, the formation of several specific protein-protein complexes appears to be key in determining whether apoptosis is triggered. Likewise, in another key apoptotic pathway, sometimes referred to as the “death receptor pathway,” the protein, NF- $\kappa$ B, either may be sequestered in a complex with protein, I- $\kappa$ B, or it may in a free state that permits it to initiate transcriptional events in the nucleus that lead to down regulation of apoptosis.

Signaling pathways have been difficult to study not only because of their complexity and interconnectedness, but also because of the disruptive procedures required for analysis of intracellular complexes, e.g. Weng et al, *Science*, 284: 92-96 (1999); Machida et al, *Molecular & Cellular Proteomics*, 2.4: 215-233 (2003); Price et al, *Methods in Molecular Biology*, 218: 255-267 (2003). A wide variety of techniques have been used to study cellular protein-protein interactions and complexes, including immunoprecipitation, chemical cross-linking, yeast two-hybrid systems, tagged fusion proteins, bioluminescence resonance energy transfer (BRET), fluorescence resonance energy transfer (FRET), mass spectroscopy, and the like, e.g. Golemis, editor, *Protein-Protein Interactions* (Cold Spring Harbor Laboratory Press, New York, 2002); Price et al (cited above); Sorkin et al, *Curr. Biol.*, 10: 1395-1398 (2000); McVey et al, *J. Biol. Chem.*, 17: 14092-14099 (2001); Salim et al, *J. Biol. Chem.*, 277: 15482-15485 (2002); Angers

et al, Proc. Natl. Acad. Sci., 97: 3684-3689 (2000); Jones et al, Proteomics, 2: 76-84 (2002); and Petricoin III, et al, The Lancet, 359: 572-577 (2002). Unfortunately, such techniques are difficult to apply, generally lack sufficient sensitivity to provide an accurate picture of the state of a signaling pathway, and/or cannot measure multiple components or interacting components that are crucial for pathway activation.

In view of the above, the availability of a convenient and sensitive assay for the activation of the mitochondrial apoptotic pathway would advance the cancer treatment field significantly, and in more particularly, would provide a much needed tool for diagnosis, patient stratification, and drug development.

#### SUMMARY OF THE INVENTION

The invention provides methods and kits for determining the apoptotic status of cells in a sample by measuring relative levels of selected protein-protein complexes characteristic of apoptotic pathways. In one aspect, the invention relates the apoptotic status of cells with the relative amounts of apoptotic pathway complexes including at least one complex selected from the group consisting of a complex comprising a Bcl-2 protein and a BH3-only protein, a complex comprising a 14-3-3 protein and a BAD protein, and a complex comprising an NF-kB protein and an I-kB protein. In another aspect, the invention provides a method of determining a status of a cancer in a patient by determining relative amounts in a specimen of apoptotic pathway complexes including at least one complex selected from the group consisting of a complex comprising a Bcl-2 protein and a BH3-only protein, a complex comprising a 14-3-3 protein and a BAD protein, and a complex comprising an NF-kB protein and an I-kB protein.

In another aspect, the invention provides a method of determining a status of a cancer in a patient by simultaneously determining relative amounts of apoptotic pathway complexes comprising a complex of a Bcl-2 protein and a BH3-only protein and a complex comprising a 14-3-3 protein and a BAD protein. In one embodiment, such complexes are measured using at least two reagents that are specific for different members of each complex: one member, referred to herein as a cleaving probe, has a cleavage-inducing moiety that may be induced to cleave susceptible bonds within its immediate proximity; and the other member, referred to herein as a binding compound, has one or more molecular tags attach by linkages that are cleavable by the cleavage-inducing moiety. In accordance with the embodiment, whenever such different members form a complex, the cleavable linkages are brought within the effective cleaving proximity of the cleavage-inducing moiety so that molecular tags can be released. The molecular tags are then separated from the reaction mixture and quantified to provide a measure of complex formation.

The present invention provides a method for determining the apoptotic status of cells in a sample, such as a tumor specimen from a patient. Apoptotic status is useful for determining disease status of a patient, and in some embodiments, prognosis, drug efficacy, patient responsiveness to therapy, and whether adjuvant therapy is recommended, particularly in neoplastic disease.

#### **BRIEF DESCRIPTION OF THE DRAWINGS**

Figures 1A-1E illustrate diagrammatically several embodiments of the method of the invention for measuring the presence of apoptotic complexes.

Figures 2A-2B illustrate diagrammatically methods for attaching molecular tags to antibodies.

Figures 3A-3F illustrate oxidation-labile linkages and their respective cleavage reactions mediated by singlet oxygen.

Figures 4A-4J show the structures of tags that have been designed and synthesized.

Figures 5A-5D illustrate the chemistries of synthesis of the tag moieties illustrated in Figure 6.

Figures 6A-6C diagrammatically illustrate a microfluidics device for implementing a step of electrophoretically separating molecular tags.

Figs. 7A-7C illustrate an embodiment of the invention for simultaneously measuring BAD//14-3-3 and BAD//Bcl-2 complexes.

#### **Definitions**

“14-3-3 protein” means a human protein capable of forming a stable complex with a human BAD phosphorylated at Ser-112 and/or Ser-155, such protein having an amino acid sequence substantially identical to that described under NCBI accession number AAH56867 or in Strausberg et al, Proc. Natl. Acad. Sci., 99: 16899-16903 (2002). In one aspect, a 14-3-3 protein hereunder is at least eighty percent identical, and more preferably ninety percent identical, to the amino acid described under NCBI accession number AAH56867 or in Strausberg et al, Proc. Natl. Acad. Sci., 99: 16899-16903 (2002).

“Antibody” means an immunoglobulin that specifically binds to, and is thereby defined as complementary with, a particular spatial and polar organization of another molecule. The antibody can be monoclonal or polyclonal and can be prepared by techniques that are well known in the art such as immunization of a host and collection of sera (polyclonal) or by preparing continuous hybrid cell lines and collecting the secreted protein (monoclonal), or by cloning and expressing nucleotide sequences or mutagenized versions thereof coding at least for the amino

acid sequences required for specific binding of natural antibodies. Antibodies may include a complete immunoglobulin or fragment thereof, which immunoglobulins include the various classes and isotypes, such as IgA, IgD, IgE, IgG1, IgG2a, IgG2b and IgG3, IgM, etc. Fragments thereof may include Fab, Fv and F(ab')<sub>2</sub>, Fab', and the like. In addition, aggregates, polymers, and conjugates of immunoglobulins or their fragments can be used where appropriate so long as binding affinity for a particular polypeptide is maintained.

"Antibody binding composition" means a molecule or a complex of molecules that comprises one or more antibodies, or fragments thereof, and derives its binding specificity from such antibody or antibody fragment. Antibody binding compositions include, but are not limited to, (i) antibody pairs in which a first antibody binds specifically to a target molecule and a second antibody binds specifically to a constant region of the first antibody; a biotinylated antibody that binds specifically to a target molecule and a streptavidin protein, which protein is derivatized with moieties such as molecular tags or photosensitizers, or the like, via a biotin moiety; (ii) antibodies specific for a target molecule and conjugated to a polymer, such as dextran, which, in turn, is derivatized with moieties such as molecular tags or photosensitizers, either directly by covalent bonds or indirectly via streptavidin-biotin linkages; (iii) antibodies specific for a target molecule and conjugated to a bead, or microbead, or other solid phase support, which, in turn, is derivatized either directly or indirectly with moieties such as molecular tags or photosensitizers, or polymers containing the latter.

"Antigenic determinant," or "epitope" means a site on the surface of a molecule, usually a protein, to which a single antibody molecule binds; generally a protein has several or many different antigenic determinants and reacts with antibodies of many different specificities. A preferred antigenic determinant is a phosphorylation site of a protein.

"Apoptosis," or "programmed cell death," means a process in which a cell destroys itself by a series of steps that includes the release of proteolytic enzymes, such as caspase enzymes.

"Apoptotic pathway," or equivalently "apoptotic signaling pathway," refers to a particular series of steps leading to cell destruction wherein the steps include characteristic molecular events, such as phosphorylation or de-phosphorylation of particular proteins, the formation of complexes between particular proteins, the dissociation of complexes, and the like. "Apoptotic complex"

refers to a protein-protein complex that forms as part of a particular apoptotic pathway.

"Apoptotic status" is a term of relative degree that refers to a pattern or profile of relative amounts of one or more complexes in one or more apoptotic pathways that is correlated with cell death or with cell survival. For example, an amount of 14-3-3//BAD complex in excess of an amount of Bcl-2//BAD complex in the same sample suggests cell survival; thus, the value of apoptotic status is low. Particular levels or values, or measurement scales, for apoptotic status depend on several factors, including the cell types in a sample, whether a disease condition is

present, the type of complexes being targeted, and the like. Levels or values of apoptotic status may be determined by reference to control samples that have known or normal levels of selected apoptotic complexes. As used herein, "status" in reference to a cancer patient means the apoptotic status of cells from a sample, specimen, or biopsy from a cancer patient. Such "status" may relate to disease determination or classification, prognosis, drug efficacy, patient responsiveness to therapy, whether adjuvant therapy is recommended, likelihood of recurrence of disease, or the like.

"BAD protein" means a human protein capable of forming a stable complex with a human 14-3-3 protein whenever phosphorylated at Ser-112 and/or Ser-155, and capable of forming a stable complex with a human Bcl-2 protein whenever there is no phosphate group attached to Ser-112 and Ser-155, such BAD protein having an amino acid sequence substantially identical to that described under NCBI accession number AAH01901 or in Strausberg et al, Proc. Natl. Acad. Sci., 99: 16899-16903 (2002). In one aspect, a BAD protein hereunder is at least eighty percent identical, and more preferably ninety percent identical, to the amino acid described under NCBI accession number AAH01901 or in Strausberg et al, Proc. Natl. Acad. Sci., 99: 16899-16903 (2002).

"Bcl-2 protein" means a human protein capable of forming a stable complex with a human BAD protein whenever the BAD protein has no phosphate group attached to Ser-112 or Ser-155, and having an amino acid sequence substantially identical to that described under NCBI accession number AAH17214 or in Strausberg et al, Proc. Natl. Acad. Sci., 99: 16899-16903 (2002). In one aspect, a Bcl-2 protein hereunder is at least eighty percent identical, and more preferably ninety percent identical, to the amino acid described under NCBI accession number AAH17214 or in Strausberg et al, Proc. Natl. Acad. Sci., 99: 16899-16903 (2002).

"Bcl-X<sub>L</sub> protein" means a human protein capable of forming a stable complex with a human BAD protein whenever the BAD protein has no phosphate group attached to Ser-112 or Ser-155, and having an amino acid sequence substantially identical to that described under NCBI accession number NP\_620120 or in Oltvai et al, Cell, 74: 609-619 (1993). In one aspect, a Bcl-X<sub>L</sub> protein hereunder is at least eighty percent identical, and more preferably ninety percent identical, to the amino acid described under NCBI accession number NP\_620120 or in Oltvai et al, Cell, 74: 609-619 (1993).

"BH3 only protein" means a human protein containing a BH3 domain, but not a BH1, BH2, or BH4 domains, and is capable of forming a stable complex with a Bcl-2 protein. Polypeptide domains, BH1, BH2, BH3, and BH4, are characteristic domains of the Bcl-2 family of proteins, which are described in the following references; Baell and Huang, Biochem. Pharmacology, 64: 851-863 (2002); Sattler et al, Science, 275: 983-985 (1997); Gross et al, Genes & Development, 13: 1899-1911 (1999); and Puthalakath et al, Cell Death and

Differentiation, 9: 505-512 (2002); which are incorporated by reference. A BH3 domain is represented by the following consensus sequence of 12 amino acids: "-I-A-X<sub>1</sub>-X<sub>2</sub>-L-R-R-I-G-D-E-F-," wherein X<sub>1</sub> is any amino acid and X<sub>2</sub> is a charged amino acid. Preferably, a BH3 domain comprises an amino acid sequence of the consensus sequence, or a sequence having from 1 to 4 conservative amino acid substitutions and/or deletions with respect to the consensus sequence, with the proviso that the leucine (L) in position five and the aspartic acid (D) in position 10 are not substituted or deleted. Exemplary BH3 only proteins include BID, BAD, BIK, BLK, HRK, BIM, NIP3, and NIX/BNIP3, descriptions of which are available from the National Center for Biotechnology Information (NCBI). A preferred BH3 only protein is BAD.

"Binding moiety" means any molecule to which molecular tags can be directly or indirectly attached that is capable of specifically binding to an analyte. Binding moieties include, but are not limited to, antibodies, antibody binding compositions, peptides, proteins, nucleic acids, and organic molecules having a molecular weight of up to 1000 daltons and consisting of atoms selected from the group consisting of hydrogen, carbon, oxygen, nitrogen, sulfur, and phosphorus. Preferably, binding moieties are antibodies or antibody binding compositions.

"Capillary-sized" in reference to a separation column means a capillary tube or channel in a plate or microfluidics device, where the diameter or largest dimension of the separation column is between about 25-500 microns, allowing efficient heat dissipation throughout the separation medium, with consequently low thermal convection within the medium.

"Chromatography" or "chromatographic separation" as used herein means or refers to a method of analysis in which the flow of a mobile phase, usually a liquid, containing a mixture of compounds, e.g. molecular tags, promotes the separation of such compounds based on one or more physical or chemical properties by a differential distribution between the mobile phase and a stationary phase, usually a solid. The one or more physical characteristics that form the basis for chromatographic separation of analytes, such as molecular tags, include but are not limited to molecular weight, shape, solubility, pKa, hydrophobicity, charge, polarity, and the like. In one aspect, as used herein, "high pressure (or performance) liquid chromatography" ("HPLC") refers to a liquid phase chromatographic separation that (i) employs a rigid cylindrical separation column having a length of up to 300 mm and an inside diameter of up to 5 mm, (ii) has a solid phase comprising rigid spherical particles (e.g. silica, alumina, or the like) having the same diameter of up to 5  $\mu$ m packed into the separation column, (iii) takes place at a temperature in the range of from 35°C to 80°C and at column pressure up to 150 bars, and (iv) employs a flow rate in the range of from 1  $\mu$ L/min to 4 mL/min. Preferably, solid phase particles for use in HPLC are further characterized in (i) having a narrow size distribution about the mean particle diameter, with substantially all particle diameters being within 10% of the mean, (ii) having the same pore size in the range of from 70 to 300 angstroms, (iii) having a surface area in the range of from 50

to 250 m<sup>2</sup>/g, and (iv) having a bonding phase density (i.e. the number of retention ligands per unit area) in the range of from 1 to 5 per nm<sup>2</sup>. Exemplary reversed phase chromatography media for separating molecular tags include particles, e.g. silica or alumina, having bonded to their surfaces retention ligands, such as phenyl groups, cyano groups, or aliphatic groups selected from the group including C<sub>8</sub> through C<sub>18</sub>. Chromatography in reference to the invention includes “capillary electrochromatography” (“CEC”), and related techniques. CEC is a liquid phase chromatographic technique in which fluid is driven by electroosmotic flow through a capillary-sized column, e.g. with inside diameters in the range of from 30 to 100 μm. CEC is disclosed in Svec, Adv. Biochem. Eng. Biotechnol. 76: 1-47 (2002); Vanhoenacker et al, Electrophoresis, 22: 4064-4103 (2001); and like references. CEC column may use the same solid phase materials as used in conventional reverse phase HPLC and additionally may use so-called “monolithic” non-particular packings. In some forms of CEC, pressure as well as electroosmosis drives an analyte-containing solvent through a column.

“Complex” as used herein means an assemblage or aggregate of molecules in direct or indirect contact with one another. As used herein, “contact,” or more particularly, “direct contact” in reference to a complex of molecules, or in reference to specificity or specific binding means two molecules are close enough that weak noncovalent chemical interactions, such as Van der Waal forces, hydrogen bonding, ionic and hydrophobic interactions, and the like, dominate the interaction of the molecules. Generally, a complex of molecules is stable in that under assay conditions the complex is thermodynamically more favorable than a non-aggregated state of its component molecules. Preferably, as used herein, “complex” refers to a stable aggregate of two or more proteins. More preferably, a complex is a stable aggregate comprising from 2 to 4 or from 2 to 6 proteins.

“I-kB protein,” or “inhibitor of NF-kB protein,” means a human protein capable of forming a stable complex with a human NF-kB protein whenever the I-kB protein is in a partially phosphorylated state, and having an amino acid sequence substantially identical to that described under NCBI accession number O00221 or in Baeuerle and Baltimore, Science, 242: 540-546 (1988). In one aspect, an I-kB protein hereunder is at least eighty percent identical, and more preferably ninety percent identical, to the amino acid described under NCBI accession number O00221 or in Baeuerle and Baltimore, Science, 242: 540-546 (1988).

The term “isolated” in reference to a polypeptide or protein means substantially separated from the components of its natural environment. Preferably, an isolated polypeptide or protein is a composition that consists of at least eighty percent of the polypeptide or protein identified by sequence on a weight basis as compared to components of its natural environment; more preferably, such composition consists of at least ninety-five percent of the polypeptide or protein identified by sequence on a weight basis as compared to components of its natural

environment; and still more preferably, such composition consists of at least ninety-nine percent of the polypeptide or protein identified by sequence on a weight basis as compared to components of its natural environment. Most preferably, an isolated polypeptide or protein is a homogeneous composition that can be resolved as a single spot after conventional separation by two-dimensional gel electrophoresis based on molecular weight and isoelectric point. Protocols for such analysis by conventional two-dimensional gel electrophoresis are well known to one of ordinary skill in the art, e.g. Hames and Rickwood, Editors, *Gel Electrophoresis of Proteins: A Practical Approach* (IRL Press, Oxford, 1981); Scopes, *Protein Purification* (Springer-Verlag, New York, 1982); Rabilloud, Editor, *Proteome Research: Two-Dimensional Gel Electrophoresis and Identification Methods* (Springer-Verlag, Berlin, 2000).

As used herein, the term "kit" refers to any delivery system for delivering materials. In the context of reaction assays, such delivery systems include systems that allow for the storage, transport, or delivery of reaction reagents (e.g., probes, enzymes, etc. in the appropriate containers) and/or supporting materials (e.g., buffers, written instructions for performing the assay etc.) from one location to another. For example, kits include one or more enclosures (e.g., boxes) containing the relevant reaction reagents and/or supporting materials. Such contents may be delivered to the intended recipient together or separately. For example, a first container may contain an enzyme for use in an assay, while a second container contains probes.

"NF-kB protein," or "nuclear factor kappa B protein," means a human protein capable of forming a stable complex with a human I-kB protein whenever the I-kB protein is in a partially phosphorylated state, and having an amino acid sequence substantially identical to that described under NCBI accession number NP\_003989. In one aspect, an NF-kB protein hereunder is at least eighty percent identical, and more preferably ninety percent identical, to the amino acid described under NCBI accession number NP\_003989.

The term "percent identical," or like term, used in respect of the comparison of a reference sequence and another sequence (i.e. a "candidate" sequence, means that in an optimal alignment between the two sequences, the candidate sequence is identical to the reference sequence in a number of subunit positions equivalent to the indicated percentage, the subunits being nucleotides for polynucleotide comparisons or amino acids for polypeptide comparisons. As used herein, an "optimal alignment" of sequences being compared is one that maximizes matches between subunits and minimizes the number of gaps employed in constructing an alignment. Percent identities may be determined with commercially available implementations of algorithms described by Needleman and Wunsch, *J. Mol. Biol.*, 48: 443-453 (1970) ("GAP" program of Wisconsin Sequence Analysis Package, Genetics Computer Group, Madison, WI). Other software packages in the art for constructing alignments and calculating percentage identity or other measures of similarity include the "BestFit" program, based on the algorithm of

Smith and Waterman, *Advances in Applied Mathematics*, 2: 482-489 (1981) (Wisconsin Sequence Analysis Package, Genetics Computer Group, Madison, WI). In other words, for example, to obtain a polypeptide having an amino acid sequence at least 95 percent identical to a reference amino acid sequence, up to five percent of the amino acid residues in the reference sequence may be deleted or substituted with another amino acid, or a number of amino acids up to five percent of the total amino acid residues in the reference sequence may be inserted into the reference sequence. These alterations of the reference sequence may occur at the amino or carboxy terminal positions of the reference amino acid sequence or anywhere between those terminal positions, interspersed either individually among residues in the reference sequence or in one or more contiguous groups with in the references sequence. It is understood that in making comparisons with reference sequences of the invention that candidate sequence may be a component or segment of a larger polypeptide or polynucleotide and that such comparisons for the purpose computing percentage identity is to be carried out with respect to the relevant component or segment.

“Polypeptide” refers to a class of compounds composed of amino acid residues chemically bonded together by amide linkages with elimination of water between the carboxy group of one amino acid and the amino group of another amino acid. A polypeptide is a polymer of amino acid residues, which may contain a large number of such residues. Peptides are similar to polypeptides, except that, generally, they are comprised of a lesser number of amino acids. Peptides are sometimes referred to as oligopeptides. There is no clear-cut distinction between polypeptides and peptides. For convenience, in this disclosure and claims, the term “polypeptide” will be used to refer generally to peptides and polypeptides. The amino acid residues may be natural or synthetic.

“Protein” refers to a polypeptide, usually synthesized by a biological cell, folded into a defined three-dimensional structure. Proteins are generally from about 5,000 to about 5,000,000 or more in molecular weight, more usually from about 5,000 to about 1,000,000 molecular weight, and may include posttranslational modifications, such as acetylation, acylation, ADP-ribosylation, amidation, covalent attachment of flavin, covalent attachment of a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of phosphatidylinositol, cross-linking, cyclization, disulfide bond formation, farnesylation, demethylation, formation of covalent cross-links, formation of cystine, formation of pyroglutamate, formylation, gamma-carboxylation, glycosylation, GPI anchor formation, hydroxylation, iodination, methylation, myristoylation, oxidation, phosphorylation, prenylation, racemization, selenoylation, sulfation, and ubiquitination, e.g.

Wold, F., *Post-translational Protein Modifications: Perspectives and Prospects*, pgs. 1-12 in *Post-translational Covalent Modification of Proteins*, B. C. Johnson, Ed., Academic Press, New York,

1983. Proteins include, by way of illustration and not limitation, cytokines or interleukins, enzymes such as, *e.g.*, kinases, proteases, galactosidases and so forth, protamines, histones, albumins, immunoglobulins, scleroproteins, phosphoproteins, mucoproteins, chromoproteins, lipoproteins, nucleoproteins, glycoproteins, T-cell receptors, proteoglycans, and the like.

5           The term “sample” means a quantity of material that is suspected of containing one or more molecular complexes that are to be detected or measured. As used herein, the term includes a specimen (*e.g.*, a biopsy or medical specimen) or a culture (*e.g.*, microbiological culture). It also includes both biological and environmental samples. A sample may include a specimen of synthetic origin. Biological samples may be animal, including human, fluid, solid  
10 (*e.g.*, stool) or tissue, as well as liquid and solid food and feed products and ingredients such as dairy items, vegetables, meat and meat by-products, and waste. Biological samples may include materials taken from a patient including, but not limited to cultures, blood, saliva, cerebral spinal fluid, pleural fluid, milk, lymph, sputum, semen, needle aspirates, and the like. Biological samples may be obtained from all of the various families of domestic animals, as well as feral or  
15 wild animals, including, but not limited to, such animals as ungulates, bear, fish, rodents, etc. Environmental samples include environmental material such as surface matter, soil, water and industrial samples, as well as samples obtained from food and dairy processing instruments, apparatus, equipment, utensils, disposable and non-disposable items. These examples are not to be construed as limiting the sample types applicable to the present invention. In particular,  
20 biological samples include fixed biological specimens, such as patient biopsy specimens treated with a fixative, biological specimens embedded in paraffin, frozen biological specimens, smears, and the like.

A “separation profile” in reference to the separation of molecular tags means a chart, graph, curve, bar graph, or other representation of signal intensity data versus a parameter related  
25 to the molecular tags, such as retention time, mass, or the like, that provides a readout, or measure, of the number of molecular tags of each type produced in an assay. A separation profile may be an electropherogram, a chromatogram, an electrochromatogram, a mass spectrogram, or like graphical representation of data depending on the separation technique employed. A “peak” or a “band” or a “zone” in reference to a separation profile means a region  
30 where a separated compound is concentrated. There may be multiple separation profiles for a single assay if, for example, different molecular tags have different fluorescent labels having distinct emission spectra and data is collected and recorded at multiple wavelengths. In one aspect, released molecular tags are separated by differences in electrophoretic mobility to form an electropherogram wherein different molecular tags correspond to distinct peaks on the  
35 electropherogram. A measure of the distinctness, or lack of overlap, of adjacent peaks in an electropherogram is “electrophoretic resolution,” which may be taken as the distance between

adjacent peak maximums divided by four times the larger of the two standard deviations of the peaks. Preferably, adjacent peaks have a resolution of at least 1.0, and more preferably, at least 1.5, and most preferably, at least 2.0. In a given separation and detection system, the desired resolution may be obtained by selecting a plurality of molecular tags whose members have electrophoretic mobilities that differ by at least a peak-resolving amount, such quantity depending on several factors well known to those of ordinary skill, including signal detection system, nature of the fluorescent moieties, the diffusion coefficients of the tags, the presence or absence of sieving matrices, nature of the electrophoretic apparatus, *e.g.* presence or absence of channels, length of separation channels, and the like.

“Specific” or “specificity” in reference to the binding of one molecule to another molecule, such as a binding compound, or probe, for a target analyte or complex, means the recognition, contact, and formation of a stable complex between the probe and target, together with substantially less recognition, contact, or complex formation of the probe with other molecules. In one aspect, “specific” in reference to the binding of a first molecule to a second molecule means that to the extent the first molecule recognizes and forms a complex with another molecules in a reaction or sample, it forms the largest number of the complexes with the second molecule. In one aspect, this largest number is at least fifty percent of all such complexes form by the first molecule. Generally, molecules involved in a specific binding event have areas on their surfaces or in cavities giving rise to specific recognition between the molecules binding to each other. Examples of specific binding include antibody-antigen interactions, enzyme-substrate interactions, formation of duplexes or triplexes among polynucleotides and/or oligonucleotides, receptor-ligand interactions, and the like.

As used herein, the term “spectrally resolvable” in reference to a plurality of fluorescent labels means that the fluorescent emission bands of the labels are sufficiently distinct, *i.e.* sufficiently non-overlapping, that molecular tags to which the respective labels are attached can be distinguished on the basis of the fluorescent signal generated by the respective labels by standard photodetection systems, *e.g.* employing a system of band pass filters and photomultiplier tubes, or the like, as exemplified by the systems described in U.S. Pat. Nos. 4,230,558; 4,811,218, or the like, or in Wheeless et al, pgs. 21-76, in *Flow Cytometry: Instrumentation and Data Analysis* (Academic Press, New York, 1985).

As used herein, “tagged probe” is used synonymously with “binding compound.”

#### DETAILED DESCRIPTION OF THE INVENTION

In one aspect, the invention includes a method for determining apoptotic status of cells in a sample by simultaneously measuring the presence, absence, and/or relative quantities of selected protein-protein complexes in major apoptotic pathways. Preferably, such complexes are

measured using binding compounds having one or more molecular tags releasably attached, such that after binding to a protein in a complex, the molecular tags may be released and separated from the reaction, or assay, mixture.

In one aspect, the invention includes a method for detecting or measuring the amounts of one or more complexes comprising the following steps: (i) providing for each of the one or more complexes a cleaving probe specific for a first protein in each of the one or more complexes, each cleaving probe having a cleavage-inducing moiety with an effective proximity; (ii) providing one or more binding compounds specific for a second protein of each of the one or more complexes, such that each binding compound has one or more molecular tags each attached thereto by a cleavable linkage, and such that the one or more molecular tags attached to different binding compounds have different separation characteristics so that upon separation molecular tags from different binding compounds form distinct peaks in a separation profile; (iii) mixing the cleaving probes, the binding compounds, and the one or more complexes such that cleaving probes specifically bind to first proteins of the complexes and binding compounds specifically bind to the second proteins of the complexes and such that cleavable linkages of the binding compounds are within the effective proximity of cleavage-inducing moieties of the cleaving probes so that molecular tags are released; and (iv) separating and identifying the released molecular tags to determine the presence or absence or the amount of the one or more complexes of proteins. Preferably, complexes and first and second proteins are selected from the complexes listed in Table III.

The invention provides methods of detecting or measuring amounts apoptotic complexes in a sample by the use of compositions comprising cleaving probes that generate a locally acting cleaving agent and binding compounds labeled with releasable molecular tags that are released by the cleaving agent. Complex formation is detected by designing cleaving probes and binding compounds such that at least one cleaving probe specifically binds to a different component of a complex than at least one of the binding compounds. In this manner, molecular tags of a predetermined type are released only when a complex is formed.

The operation of one embodiment of the invention is illustrated in Fig. 1A. Molecular complex (100) forms by the binding of proteins (104) and (102), e.g. 14-3-3 and phosphorylated BAD. Reagents (107) of the invention, comprising cleaving probes (108) (in this illustration having photosensitizer "PS" attached) and binding compounds (106), are mixed (109) with a sample containing complex (100) under conditions that permit the specific binding (112) of cleaving probes (108) and binding compounds (106) to their respective antigenic determinants on complex (100) that are on different proteins of the complex. After binding, and optionally washing or buffer exchange, cleaving probes (108) are activated to generate an active species that, e.g. in the case of singlet oxygen, diffuses out from a photosensitizers to an effective

proximity (110). Cleavable linkages within this proximity are cleaved and molecular tags are released (114). Released molecular tags (116) are then separated (117) and a separation profile (120), such as an electropherogram, is produced, in which peak (118) is identified and correlated to molecular tag, "mT<sub>1</sub>." By employing additional binding compounds and molecular tags, additional complexes may be measured. A more complex embodiment is illustrated in Fig. 1B, in which an additional binding compound is employed to give a measure of the total amount of protein (104) in a sample. Reagents (122) of the invention comprise (i) cleaving probes (108), first binding compound (106), and second binding compound (107), wherein first binding compound (106) is specific for protein (102) and second binding compound (107) is specific for protein (104) at a different antigenic determinant than that cleaving probe (108) is specific for. As with the embodiment of Fig. 1A, after binding of the reagents, cleaving probe (108) is activated to produce active species that cleave the cleavable linkages of the molecular tags within the effective proximity of the photosensitizer. In this embodiment, molecular tags are released from monomers of protein (104) that have both reagents (107) and (108) attached and from heterodimers that have reagent (108) attached and either or both of reagents (106) and (107) attached. Released molecular tags (123) are separated, and peaks (118 and 124) in a separation profile (126) are correlated to the amounts of the released molecular tags. In this embodiment, relative peak heights, or areas, may reflect (i) the differences in affinity of the first and second binding compounds for their respective antigenic determinants, and/or (ii) the presence or absence of the antigenic determinant that the binding compound is specific for. The later situation is important whenever a binding compound is used to monitor the post-translational state of a protein, e.g. phosphorylation state.

Homodimeric as well as heterodimeric complexes of apoptotic signaling molecules may be measured as illustrated in Fig. 1A-1C. Fig. 1C illustrates one approach for measuring homodimeric complexes. As above, an assay may comprise three reagents (128): cleaving probes (134), first binding compound (130), and second binding compound (132). First binding compound (130) and cleaving probe (134) are constructed to be specific for the same antigenic determinant (135) on protein (138) that exists (140) in a sample as either a homodimer (136) or a monomer (138). After reagents (128) are combined with a sample under conditions that promote the formation of stable complexes between the reagents and their respective targets, multiple complexes (142 through 150) form in the assay mixture. Because cleaving probe (134) and binding compound (130) are specific for the same antigenic determinant (135), four different combinations (144 through 150) of reagents may form complexes with homodimers. Of the complexes in the assay mixture, only those (143) with both a cleaving probe (134) and at least one binding compound will contribute released molecular tags (151) for separation and detection (154). In this embodiment, the size of peak (153) is proportional to the amount of homodimer in

the assay mixture, while the size of peak (152) is proportional to the total amount of protein (138) in the assay mixture, both in monomeric form (142) or in homodimeric form (146 and 148).

Another aspect of the invention is illustrated in Figs. 1D and 1E, which provides for the simultaneous detection or measurement of multiple complexes in a cellular sample. Cells (160), which may be from a sample from in vitro cultures or from a specimen of patient tissue, are lysed (172) to render accessible molecular complexes associated with the cell membrane, and/or within the cytosol, and/or within the cell nucleus. Complexes associated with apoptotic signaling include, but are not limited to, surface receptor complexes, such as receptor dimers, receptor complexes including adaptor or scaffold molecules of various types, dimers and higher order complexes of intracellular proteins, phosphorylation sites of proteins in such complexes, and the like. After lysing, the resulting lysate (174) is combined with assay reagents (176) that include multiple cleaving probes (175) and multiple binding compounds (177). Assay conditions are selected (178) that allow reagents (176) to specifically bind to their respective targets, so that upon activation cleavable linkages within the effective proximity (180) of the cleavage-inducing moieties are cleaved and molecular tags are released (182). As above, after cleavage, the released molecular tags are separated (184) and identified in a separation profile (186), such as an electropherogram, and based on the number and quantities of molecular tags measured, a profile is obtained of the selected molecular complexes in the cells of the sample.

#### Preparation of Samples

Samples containing molecular complexes may come from a wide variety of sources including cell cultures, animal or plant tissues, microorganisms, patient biopsies, or the like. Samples are prepared for assays of the invention using conventional techniques, which may depend on the source from which a sample is taken. Guidance for preparing cell membranes for analysis can be found in standard treatises, such as Sambrook et al, Molecular Cloning, Second Edition (Cold Spring Harbor Laboratory Press, New York, 1989); Innis et al, editors, PCR Protocols (Academic Press, New York, 1990); Berger and Kimmel, "Guide to Molecular Cloning Techniques," Vol. 152, Methods in Enzymology (Academic Press, New York, 1987); Ohlendieck, K. (1996). Protein Purification Protocols; Methods in Molecular Biology, Humana Press Inc., Totowa, NJ. Vol 59: 293-304; Method Booklet 5, "Signal Transduction" (Biosource International, Camarillo, CA, 2002); or the like. For mammalian tissue culture cells, or like sources, samples of complexes may be prepared by conventional cell lysis techniques (e.g. 0.14 M NaCl, 1.5 mM MgCl<sub>2</sub>, 10 mM Tris-Cl (pH 8.6), 0.5% Nonidet P-40, and protease and/or phosphatase inhibitors as required). For biopsies and medical specimens: Bancroft JD & Stevens A, eds. Theory and Practice of Histological Techniques (Churchill Livingstone,

Edinburgh, 1977); Pearse, Histochemistry. Theory and applied. 4<sup>th</sup> ed. (Churchill Livingstone, Edinburgh, 1980)

As described more fully below, molecular complexes in a sample are determined by separation and identification of the released molecular tags. A wide variety of separation techniques may be employed that can distinguish molecules based on one or more physical, chemical, or optical differences among molecules being separated including but not limited to electrophoretic mobility, molecular weight, shape, solubility, pKa, hydrophobicity, charge, charge/mass ratio, polarity, or the like. In one aspect, molecular tags in a plurality differ in electrophoretic mobility and optical detection characteristics and are separated by electrophoresis. In another aspect, molecular tags in a plurality differ in molecular weight, shape, solubility, pKa, hydrophobicity, charge, polarity, and are separated by normal phase or reverse phase HPLC, ion exchange HPLC, capillary electrochromatography, mass spectroscopy, gas phase chromatography, or like technique.

Another aspect of the present invention is providing sets of molecular tags that may be separated into distinct bands or peaks by the separation technique employed after they are released from binding compounds. Molecular tags within a set may be chemically diverse; however, for convenience, sets of molecular tags are usually chemically related. For example, they may all be peptides, or they may consist of different combinations of the same basic building blocks or monomers, or they may be synthesized using the same basic scaffold with different substituent groups for imparting different separation characteristics, as described more fully below. The number of molecular tags in a plurality may vary depending on several factors including the mode of separation employed, the labels used on the molecular tags for detection, the sensitivity of the binding moieties, the efficiency with which the cleavable linkages are cleaved, and the like. In one aspect, the number of molecular tags in a plurality ranges from 2 to several tens, e.g. 30. In other aspects, the size of the plurality may be in the range of from 2 to 20, 2 to 10, 3 to 20, 3 to 10, 4 to 30, 4 to 10, 5 to 20, or 5 to 10.

#### Molecular Tags and Cleavable Linkages

In one aspect, the invention provides binding compounds that have molecular tags attached via cleavable linkages. In an assay, the molecular tags are cleaved by a cleaving agent that reacts with the cleavable linkages to release the molecular tags from their respective binding compounds. The released molecular tags are then separated and detected. A wide variety of cleavable linkages and corresponding cleaving agents may be employed in the invention. Whenever a homogeneous assay format is desired, preferably, the cleaving agent is a locally acting agent. That is, as explained more fully below, a cleavage-inducing moiety is employed in the assay that may be induced to create local conditions for cleavage of the cleavable linkages.

In one aspect, such a cleavage-inducing moiety is a sensitizer that generates an active species, as described more fully below. Assays of the invention may also be conducted in a heterogeneous, or non-homogeneous, format. In such a format, binding compounds are combined with a sample in an assay reaction mixture so that the binding compound can specifically bind to their target molecules, or antigenic determinants, whenever they are available to form a stable complexes. Unbound binding compounds are then removed, or separated from, the stable complexes by washing, by filtration, centrifugation, magnetic separation, or the like. In a this format, cleavage of the molecular tags from the stable complexes need not be proximity dependent, since unbound binding compounds have been removed. Therefore, a larger variety of cleavage protocols can be used. Cleavage may still employ a sensitizer, as described above, to cleave an oxidatively labile linkage, but it may also employ various types of chemical, photochemical, or enzymatic cleavage of a variety of cleavable linking groups, such as are known in the art. For example, non-limiting examples of chemically cleavable linkages include disulfides (cleavable by reduction, typically using dithiothreitol), azo groups (cleavable with dithionate), sulfones (cleavable with basic phosphate, with or without dithiothreitol), glycols, cleavable by periodate, and esters, cleavable by hydrolysis. Photolabile linkers include, for example, azo linkages and o-nitrobenzyl ethers.

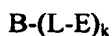
After washing, the stable complexes of binding compounds and targets may be combined with a solvent into which the molecular tags are released. Depending on the nature of the cleavable bond and the method of cleavage, the solvent may include any additional reagents for the cleavage. Where reagents for cleavage are not required, the solvent is conveniently an separation buffer, such as an electrophoretic separation medium. For example, where the cleavable linkage is photolabile, the medium may be irradiated with light of appropriate wavelength to release the molecular tags into the buffer or medium.

In either format, if the assay reaction conditions interfere with the separation technique employed, it may be necessary to remove, or exchange, the assay reaction buffer prior to cleavage and separation of the molecular tags. For example, in some embodiments, assay conditions include salt concentrations (e.g. required for specific binding) that degrade separation performance when molecular tags are separated on the basis of electrophoretic mobility.

Guidance for selecting cleaving agents, molecular tags, cleavable linkages, and other components for homogeneous or heterogeneous assay formats is disclosed in the following references: International patent publications WO 00/66607; WO 01/83502; WO 02/95356; WO 03/06947; and U.S. patents 6,322,980 and 6,514,700.

As mentioned above, an aspect of the invention includes providing mixtures of pluralities of different binding compounds, wherein each different binding compound has one or more molecular tags attached through cleavable linkages. The nature of the binding compound, cleavable linkage and molecular tag may vary widely. A binding compound may comprise an

antibody binding composition, an antibody, a peptide, a peptide or non-peptide ligand for a cell surface receptor, a protein, an oligonucleotide, an oligonucleotide analog, such as a peptide nucleic acid, a lectin, or any other molecular entity that is capable of specific binding or stable complex formation with an analyte of interest, such as a complex of proteins. In one aspect, a binding compound, which can be represented by the formula below, comprises one or more molecular tags attached to a binding moiety.



wherein B is binding moiety; L is a cleavable linkage; and E is a molecular tag. Preferably, in homogeneous assays for non-polynucleotide analytes, cleavable linkage, L, is an oxidation-labile linkage, and more preferably, it is a linkage that may be cleaved by singlet oxygen. The moiety “-(L-E)<sub>k</sub>” indicates that a single binding compound may have multiple molecular tags attached via cleavable linkages. In one aspect, k is an integer greater than or equal to one, but in other embodiments, k may be greater than several hundred, e.g. 100 to 500, or k is greater than several hundred to as many as several thousand, e.g. 500 to 5000. Within a composition of the invention, usually each of the plurality of different types of binding compound has a different molecular tag, E. Cleavable linkages, e.g. oxidation-labile linkages, and molecular tags, E, are attached to B by way of conventional chemistries.

Preferably, B is an antibody binding composition. Such compositions are readily formed from a wide variety of commercially available antibodies, both monoclonal and polyclonal, specific for proteins of interest. In particular, antibodies specific for epidermal growth factor receptors are disclosed in the following patents, which are incorporated by references: 5,677,171; 5,772,997; 5,968,511; 5,480,968; 5,811,098. U.S. patent 6,488,390, incorporated herein by reference, discloses antibodies specific for a G-protein coupled receptor, CCR4. U.S. patent 5,599,681, incorporated herein by reference, discloses antibodies specific for phosphorylation sites of proteins. Commercial vendors, such as Cell Signaling Technology (Beverly, MA), Biosource International (Camarillo, CA), and Upstate (Charlottesville, VA), also provide monoclonal and polyclonal antibodies specific for many proteins in apoptotic pathways, including proteins listed in Table II.

When L is oxidation labile, L may be a thioether or its selenium analog; or an olefin, which contains carbon-carbon double bonds, wherein cleavage of a double bond to an oxo group, releases the molecular tag, E. Illustrative thioether bonds are disclosed in Willner et al, U.S. patent 5,622,929 which is incorporated by reference. Illustrative olefins include vinyl sulfides, vinyl ethers, enamines, imines substituted at the carbon atoms with an  $\alpha$ -methine (CH, a carbon atom having at least one hydrogen atom), where the vinyl group may be in a ring, the heteroatom

may be in a ring, or substituted on the cyclic olefinic carbon atom, and there will be at least one and up to four heteroatoms bonded to the olefinic carbon atoms. The resulting dioxetane may decompose spontaneously, by heating above ambient temperature, usually below about 75°C, by reaction with acid or base, or by photo-activation in the absence or presence of a photosensitizer.

Such linkages and reactions are described in the following exemplary references: U.S. Patent Nos. 5,756,726; 5,800,999; and 5,886,238.

Exemplary cleavable linkages and their cleavage products are illustrated in Figures 3 A-F. The thiazole cleavable linkage, “-CH<sub>2</sub>-thiazole-(CH<sub>2</sub>)<sub>n</sub>-C(=O)-NH-protein,” shown in Fig. 3A, results in an molecular tag with the moiety “-CH<sub>2</sub>-C(=O)-NH-CHO.” Preferably, n is in the range of from 1 to 12, and more preferably, from 1 to 6. The oxazole cleavable linkage, “-CH<sub>2</sub>-oxazole-(CH<sub>2</sub>)<sub>n</sub>-C(=O)-NH-protein,” shown in Fig. 3B, results in an molecular tag with the moiety “-CH<sub>2</sub>-C(=O)O-CHO.” An olefin cleavable linkage (Fig. 3C) is shown in connection with the binding compound embodiment “B-L-M-D,” described above and with D being a detection moiety, such as a fluorescein dye. The olefin cleavable linkage may be employed in other embodiments also. Cleavage of the illustrated olefin linkage results in an molecular tag of the form: “R-(C=O)-M-D,” where “R” may be any substituent within the general description of the molecular tags, E, provided above. Preferably, R is an electron-donating group, *e.g.* Ullman et al, U.S. patent 6,251,581; Smith and March, March’s Advanced Organic Chemistry: Reactions, Mechanisms, and Structure, 5<sup>th</sup> Edition (Wiley-Interscience, New York, 2001); and the like. More preferably, R is an electron-donating group having from 1-8 carbon atoms and from 0 to 4 heteroatoms selected from the group consisting of O, S, and N. In further preference, R is -N(Q)<sub>2</sub>, -OQ, p-[C<sub>6</sub>H<sub>4</sub>N(Q)<sub>2</sub>], furanyl, n-alkylpyrrolyl, 2-indolyl, or the like, where Q is alkyl or aryl. In further reference to the olefin cleavable linkage of Fig. 3C, substituents “X” and “R” are equivalent to substituents “X” and “Y” of the above formula describing cleavable linkage, L. In particular, X in Fig. 3C is preferably morpholino, -OR’, or -SR”, where R’ and R” are aliphatic, aromatic, alicyclic or heterocyclic having from 1 to 8 carbon atoms and 0 to 4 heteroatoms selected from the group consisting of O, S, and N. A preferred thioether cleavable linkage is illustrated in Fig. 3D having the form “-(CH<sub>2</sub>)<sub>2</sub>-S-CH(C<sub>6</sub>H<sub>5</sub>)C(=O)NH-(CH<sub>2</sub>)<sub>n</sub>-NH-,” wherein n is in the range of from 2 to 12, and more preferably, in the range of from 2 to 6. Thioether cleavable linkages of the type shown in Fig. 3D may be attached to binding moieties, T, and molecular tags, E, by way of precursor compounds shown in Figures 3E and 3F. To attach to an amino group of a binding moiety, T, the terminal hydroxyl is converted to an NHS ester by conventional chemistry. After reaction with the amino group and attachment, the Fmoc protection group is removed to produce a free amine which is then reacted with an NHS ester of the molecular tag.

Molecular tag, E, in the present invention may comprise an electrophoric tag as described in the following references when separation of pluralities of molecular tags are carried out by gas chromatography or mass spectrometry: Zhang et al, *Bioconjugate Chem.*, 13: 1002-1012 (2002); Giese, *Anal. Chem.*, 2: 165-168 (1983); and U.S. patents 4,650,750; 5,360,819; 5,516,931; 5,602,273; and the like.

Molecular tag, E, is preferably a water-soluble organic compound that is stable with respect to the active species, especially singlet oxygen, and that includes a detection or reporter group. Otherwise, E may vary widely in size and structure. In one aspect, E has a molecular weight in the range of from about 50 to about 2500 daltons, more preferably, from about 50 to about 1500 daltons. Preferred structures of E are described more fully below. E may comprise a detection group for generating an electrochemical, fluorescent, or chromogenic signal. In embodiments employing detection by mass, E may not have a separate moiety for detection purposes. Preferably, the detection group generates a fluorescent signal.

Molecular tags within a plurality are selected so that each has a unique separation characteristic and/or a unique optical property with respect to the other members of the same plurality. In one aspect, the chromatographic or electrophoretic separation characteristic is retention time under set of standard separation conditions conventional in the art, e.g. voltage, column pressure, column type, mobile phase, electrophoretic separation medium, or the like. In another aspect, the optical property is a fluorescence property, such as emission spectrum, fluorescence lifetime, fluorescence intensity at a given wavelength or band of wavelengths, or the like. Preferably, the fluorescence property is fluorescence intensity. For example, each molecular tag of a plurality may have the same fluorescent emission properties, but each will differ from one another by virtue of a unique retention time. On the other hand, or two or more of the molecular tags of a plurality may have identical migration, or retention, times, but they will have unique fluorescent properties, e.g. spectrally resolvable emission spectra, so that all the members of the plurality are distinguishable by the combination of molecular separation and fluorescence measurement.

Preferably, released molecular tags are detected by electrophoretic separation and the fluorescence of a detection group. In such embodiments, molecular tags having substantially identical fluorescence properties have different electrophoretic mobilities so that distinct peaks in an electropherogram are formed under separation conditions. Preferably, pluralities of molecular tags of the invention are separated by conventional capillary electrophoresis apparatus, either in the presence or absence of a conventional sieving matrix. Exemplary capillary electrophoresis apparatus include Applied Biosystems (Foster City, CA) models 310, 3100 and 3700; Beckman (Fullerton, CA) model P/ACE MDQ; Amersham Biosciences (Sunnyvale, CA) MegaBACE 1000 or 4000; SpectruMedix genetic analysis system; and the like. Electrophoretic

mobility is proportional to  $q/M^{2/3}$ , where  $q$  is the charge on the molecule and  $M$  is the mass of the molecule. Desirably, the difference in mobility under the conditions of the determination between the closest electrophoretic labels will be at least about 0.001, usually 0.002, more usually at least about 0.01, and may be 0.02 or more. Preferably, in such conventional apparatus, the electrophoretic mobilities of molecular tags of a plurality differ by at least one percent, and more preferably, by at least a percentage in the range of from 1 to 10 percent.

In one aspect, molecular tag,  $E$ , is  $(M, D)$ , where  $M$  is a mobility-modifying moiety and  $D$  is a detection moiety. The notation " $(M, D)$ " is used to indicate that the ordering of the  $M$  and  $D$  moieties may be such that either moiety can be adjacent to the cleavable linkage,  $L$ . That is, "B-L- $(M, D)$ " designates binding compound of either of two forms: "B-L-M-D" or "B-L-D-M."

Detection moiety,  $D$ , may be a fluorescent label or dye, a chromogenic label or dye, an electrochemical label, or the like. Preferably,  $D$  is a fluorescent dye. Exemplary fluorescent dyes for use with the invention include water-soluble rhodamine dyes, fluoresceins, 4,7-dichlorofluoresceins, benzoxanthene dyes, and energy transfer dyes, disclosed in the following references: Handbook of Molecular Probes and Research Reagents, 8<sup>th</sup> ed., (Molecular Probes, Eugene, 2002); Lee et al, U.S. patent 6,191,278; Lee et al, U.S. patent 6,372,907; Menchen et al, U.S. patent 6,096,723; Lee et al, U.S. patent 5,945,526; Lee et al, Nucleic Acids Research, 25: 2816-2822 (1997); Hobb, Jr., U.S. patent 4,997,928; Khanna *et al.*, U.S. patent 4,318,846; and the like. Preferably,  $D$  is a fluorescein or a fluorescein derivative.

The size and composition of mobility-modifying moiety,  $M$ , can vary from a bond to about 100 atoms in a chain, usually not more than about 60 atoms, more usually not more than about 30 atoms, where the atoms are carbon, oxygen, nitrogen, phosphorous, boron and sulfur. Generally, when other than a bond, the mobility-modifying moiety has from about 0 to about 40, more usually from about 0 to about 30 heteroatoms, which in addition to the heteroatoms indicated above may include halogen or other heteroatom. The total number of atoms other than hydrogen is generally fewer than about 200 atoms, usually fewer than about 100 atoms. Where acid groups are present, depending upon the pH of the medium in which the mobility-modifying moiety is present, various cations may be associated with the acid group. The acids may be organic or inorganic, including carboxyl, thionocarboxyl, thiocarboxyl, hydroxamic, phosphate, phosphite, phosphonate, phosphinate, sulfonate, sulfinate, boronic, nitric, nitrous, etc. For positive charges, substituents include amino (includes ammonium), phosphonium, sulfonium, oxonium, etc., where substituents are generally aliphatic of from about 1 - 6 carbon atoms, the total number of carbon atoms per heteroatom, usually be less than about 12, usually less than about 9. The side chains include amines, ammonium salts, hydroxyl groups, including phenolic groups, carboxyl groups, esters, amides, phosphates, heterocycles.  $M$  may be a homo-oligomer

or a hetero-oligomer, having different monomers of the same or different chemical characteristics, *e.g.*, nucleotides and amino acids.

#### Attaching Molecular Tags to Binding Moieties

Extensive guidance can be found in the literature for covalently linking molecular tags to binding compounds, such as antibodies, *e.g.* Hermanson, *Bioconjugate Techniques*, (Academic Press, New York, 1996), and the like. In one aspect of the invention, one or more molecular tags are attached directly or indirectly to common reactive groups on a binding compound. Common reactive groups include amine, thiol, carboxylate, hydroxyl, aldehyde, ketone, and the like, and may be coupled to molecular tags by commercially available cross-linking agents, *e.g.* Hermanson (cited above); Haugland, *Handbook of Fluorescent Probes and Research Products*, Ninth Edition (Molecular Probes, Eugene, OR, 2002). In one embodiment, an NHS-ester of a molecular tag is reacted with a free amine on the binding compound.

In another embodiment illustrated in Figure 2A, binding compounds comprise a biotinylated antibody (200) as a binding moiety. Molecular tags are attached to binding moiety (200) by way of avidin or streptavidin bridge (206). Preferably, in operation, binding moiety (200) is first reacted with a target complex, after which avidin or streptavidin is added (204) to form antibody-biotin-avidin complex (205). To such complexes (205) are added (208) biotinylated molecular tags (210) to form binding compound (212).

In still another embodiment illustrated in Fig. 2B, binding compounds comprise an antibody (214) derivatized with a multi-functional moiety (216) that contains multiple functional groups (218) that are reacted (220) molecular tag precursors to give a final binding compound having multiple molecular tags (222) attached. Exemplary multi-functional moieties include aminodextran, and like materials.

Once each of the binding compounds is separately derivatized by a different molecular tag, it is pooled with other binding compounds to form a plurality of binding compounds. Usually, each different kind of binding compound is present in a composition in the same proportion; however, proportions may be varied as a design choice so that one or a subset of particular binding compounds are present in greater or lower proportion depending on the desirability or requirements for a particular embodiment or assay. Factors that may affect such design choices include, but are not limited to, antibody affinity and avidity for a particular target, relative prevalence of a target, fluorescent characteristics of a detection moiety of a molecular tag, and the like.

#### Cleavage-Inducing Moiety Producing Active Species

A cleavage-inducing moiety, or cleaving agent, is a group that produces an active species that is capable of cleaving a cleavable linkage, preferably by oxidation. Preferably, the active species is a chemical species that exhibits short-lived activity so that its cleavage-inducing effects are only in the proximity of the site of its generation. Either the active species is inherently short lived, so that it will not create significant background because beyond the proximity of its creation, or a scavenger is employed that efficiently scavenges the active species, so that it is not available to react with cleavable linkages beyond a short distance from the site of its generation. Illustrative active species include singlet oxygen, hydrogen peroxide, NADH, and hydroxyl radicals, phenoxy radical, superoxide, and the like. Illustrative quenchers for active species that cause oxidation include polyenes, carotenoids, vitamin E, vitamin C, amino acid-pyrrole N-conjugates of tyrosine, histidine, and glutathione, and the like, *e.g.* Beutner et al, *Meth. Enzymol.*, 319: 226-241 (2000).

An important consideration for the cleavage-inducing moiety and the cleavable linkage is that they not be so far removed from one another when bound to a target protein that the active species generated by the sensitizer diffuses and loses its activity before it can interact with the cleavable linkage. Accordingly, a cleavable linkage preferably are within 1000 nm, preferably 20-200 nm of a bound cleavage-inducing moiety. This effective range of a cleavage-inducing moiety is referred to herein as its "effective proximity."

Generators of active species include enzymes, such as oxidases, such as glucose oxidase, xanthene oxidase, D-amino acid oxidase, NADH-FMN oxidoreductase, galactose oxidase, glyceryl phosphate oxidase, sarcosine oxidase, choline oxidase and alcohol oxidase, that produce hydrogen peroxide, horse radish peroxidase, that produces hydroxyl radical, various dehydrogenases that produce NADH or NADPH, urease that produces ammonia to create a high local pH.

A sensitizer is a compound that can be induced to generate a reactive intermediate, or species, usually singlet oxygen. Preferably, a sensitizer used in accordance with the invention is a photosensitizer. Other sensitizers included within the scope of the invention are compounds that on excitation by heat, light, ionizing radiation, or chemical activation will release a molecule of singlet oxygen. The best known members of this class of compounds include the endoperoxides such as 1,4-bis(carboxyethyl)-1,4-naphthalene endoperoxide, 9,10-diphenylanthracene-9,10-endoperoxide and 5,6,11,12-tetraphenyl naphthalene 5,12-endoperoxide. Heating or direct absorption of light by these compounds releases singlet oxygen. Further sensitizers are disclosed in the following references: Di Mascio et al, *FEBS Lett.*, 355: 287 (1994)(peroxidases and oxygenases); Kanofsky, *J.Biol. Chem.* 258: 5991-5993 (1983)(lactoperoxidase); Pierlot et al, *Meth. Enzymol.*, 319: 3-20 (2000)(thermal lysis of endoperoxides); and the like.

Attachment of a binding agent to the cleavage-inducing moiety may be direct or indirect, covalent or non-covalent and can be accomplished by well-known techniques, commonly available in the literature. See, for example, "Immobilized Enzymes," Ichiro Chibata, Halsted Press, New York (1978); Cuatrecasas, J. Biol. Chem., 245:3059 (1970). A wide variety of functional groups are available or can be incorporated. Functional groups include carboxylic acids, aldehydes, amino groups, cyano groups, ethylene groups, hydroxyl groups, mercapto groups, and the like. The manner of linking a wide variety of compounds is well known and is amply illustrated in the literature (see above). The length of a linking group to a binding agent may vary widely, depending upon the nature of the compound being linked, the effect of the distance on the specific binding properties and the like.

The cleavage-inducing moiety may be associated with the support by being covalently or non-covalently attached to the surface of the support or incorporated into the body of the support. Linking to the surface may be accomplished as discussed above. The cleavage-inducing moiety may be incorporated into the body of the support either during or after the preparation of the support. In general, the cleavage-inducing moiety is associated with the support in an amount necessary to achieve the necessary amount of active species. Generally, the amount of cleavage-inducing moiety is determined empirically.

#### Photosensitizers as Cleavage-Inducing Moieties

As mentioned above, the preferred cleavage-inducing moiety in accordance with the present invention is a photosensitizer that produces singlet oxygen. As used herein, "photosensitizer" refers to a light-adsorbing molecule that when activated by light converts molecular oxygen into singlet oxygen. Photosensitizers may be attached directly or indirectly, via covalent or non-covalent linkages, to the binding agent of a class-specific reagent. Guidance for constructing of such compositions, particularly for antibodies as binding agents, available in the literature, e.g. in the fields of photodynamic therapy, immunodiagnostics, and the like. The following are exemplary references: Ullman, *et al.*, Proc. Natl. Acad. Sci. USA 91, 5426-5430 (1994); Strong et al, Ann. New York Acad. Sci., 745: 297-320 (1994); Yarmush et al, Crit. Rev. Therapeutic Drug Carrier Syst., 10: 197-252 (1993); Pease et al, U.S. patent 5,709,994; Ullman et al, U.S. patent 5,340,716; Ullman et al, U.S. patent 6,251,581; McCapra, U.S. patent 5,516,636; and the like.

Likewise, there is guidance in the literature regarding the properties and selection of photosensitizers suitable for use in the present invention. The following are exemplary references: Wasserman and R.W. Murray. Singlet Oxygen. (Academic Press, New York, 1979); Baumstark, Singlet Oxygen, Vol. 2 (CRC Press Inc., Boca Raton, FL 1983); and Turro, Modern Molecular Photochemistry (University Science Books, 1991).

The photosensitizers are sensitizers for generation of singlet oxygen by excitation with light. The photosensitizers include dyes and aromatic compounds, and are usually compounds comprised of covalently bonded atoms, usually with multiple conjugated double or triple bonds. The compounds typically absorb light in the wavelength range of about 200 to about 1,100 nm, usually, about 300 to about 1,000 nm, preferably, about 450 to about 950 nm, with an extinction coefficient at its absorbance maximum greater than about  $500 \text{ M}^{-1} \text{ cm}^{-1}$ , preferably, about  $5,000 \text{ M}^{-1} \text{ cm}^{-1}$ , more preferably, about  $50,000 \text{ M}^{-1} \text{ cm}^{-1}$ , at the excitation wavelength. The lifetime of an excited state produced following absorption of light in the absence of oxygen will usually be at least about 100 nanoseconds, preferably, at least about 1 millisecond. In general, the lifetime must be sufficiently long to permit cleavage of a linkage in a reagent in accordance with the present invention. Such a reagent is normally present at concentrations as discussed below. The photosensitizer excited state usually has a different spin quantum number (S) than its ground state and is usually a triplet (S=1) when the ground state, as is usually the case, is a singlet (S=0). Preferably, the photosensitizer has a high intersystem crossing yield. That is, photoexcitation of a photosensitizer usually produces a triplet state with an efficiency of at least about 10%, desirably at least about 40%, preferably greater than about 80%.

Photosensitizers chosen are relatively photostable and, preferably, do not react efficiently with singlet oxygen. Several structural features are present in most useful photosensitizers. Most photosensitizers have at least one and frequently three or more conjugated double or triple bonds held in a rigid, frequently aromatic structure. They will frequently contain at least one group that accelerates intersystem crossing such as a carbonyl or imine group or a heavy atom selected from rows 3-6 of the periodic table, especially iodine or bromine, or they may have extended aromatic structures.

A large variety of light sources are available to photo-activate photosensitizers to generate singlet oxygen. Both polychromatic and monochromatic sources may be used as long as the source is sufficiently intense to produce enough singlet oxygen in a practical time duration. The length of the irradiation is dependent on the nature of the photosensitizer, the nature of the cleavable linkage, the power of the source of irradiation, and its distance from the sample, and so forth. In general, the period for irradiation may be less than about a microsecond to as long as about 10 minutes, usually in the range of about one millisecond to about 60 seconds. The intensity and length of irradiation should be sufficient to excite at least about 0.1% of the photosensitizer molecules, usually at least about 30% of the photosensitizer molecules and preferably, substantially all of the photosensitizer molecules. Exemplary light sources include, by way of illustration and not limitation, lasers such as, *e.g.*, helium-neon lasers, argon lasers, YAG lasers, He/Cd lasers, and ruby lasers; photodiodes; mercury, sodium and xenon vapor lamps; incandescent lamps such as, *e.g.*, tungsten and tungsten/halogen; flashlamps; and the like.

By way of example, a photoactivation device disclosed in Bjornson et al, International patent publication WO 03/051669 is employed. Briefly, the photoactivation device is an array of light emitting diodes (LEDs) mounted in housing that permits the simultaneous illumination of all the wells in a 96-well plate. A suitable LED for use in the present invention is a high power GaAlAs IR emitter, such as model OD-880W manufactured by OPTO DIODE CORP. (Newbury Park, CA).

Examples of photosensitizers that may be utilized in the present invention are those that have the above properties and are enumerated in the following references: Singh and Ullman, U.S. patent 5,536,834; Li et al, U.S. patent 5,763,602; Martin et al, Methods Enzymol., 186: 635-645 (1990); Yarmush et al, Crit. Rev. Therapeutic Drug Carrier Syst., 10: 197-252 (1993); Pease et al, U.S. patent 5,709,994; Ullman et al, U.S. patent 5,340,716; Ullman et al, U.S. patent 6,251,581; McCapra, U.S. patent 5,516,636; Thetford, European patent publ. 0484027; Sessler et al, SPIE, 1426: 318-329 (1991); and the like. Exemplary photosensitizers are listed in Table I.

**Table I**  
**Exemplary Photosensitizers**

Hypocrellin A	Tetraphenylporphyrin
Hypocrellin B	Halogenated derivatives of rhodamine dyes
Hypericin	metallo-Porphyrins
Halogenated derivatives of fluorescein dyes	Phthalocyanines
Rose bengal	Naphthalocyanines
Merocyanine 540	Texaphyrin-type macrocycles
Methylene blue	Hematoporphyrin
9-Thioxanthone	9,10-Dibromoanthracene
Chlorophylls	Benzophenone
Phenaleone	Chlorin e6
Protoporphyrin	Perylene
Benzoporphyrin A monacid	Benzoporphyrin B monacid

In certain embodiments the photosensitizer moiety comprises a support, as discussed above with respect to the cleavage-inducing moiety. The photosensitizer may be associated with the support by being covalently or non-covalently attached to the surface of the support or incorporated into the body of the support as discussed above. In general, the photosensitizer is associated with the support in an amount necessary to achieve the necessary amount of singlet oxygen. Generally, the amount of photosensitizer is determined empirically. Photosensitizers used as the photosensitizer are preferably relatively non-polar to assure dissolution into a lipophilic member when the photosensitizer is incorporated in, for example, a latex particle to form photosensitizer beads, *e.g.* as disclosed by Pease et al., U.S. patent 5,709,994. For example, the photosensitizer rose bengal is covalently attached to 0.5 micron latex beads by means of

chloromethyl groups on the latex to provide an ester linking group, as described in J. Amer. Chem. Soc., 97: 3741 (1975).

In one aspect of the invention, a class-specific reagent comprises a first binding agent that is an antibody and a cleavage-inducing moiety that is a photosensitizer, such that the photosensitizer is covalently linked to the antibody, *e.g.* using well know techniques as disclosed in Strong et al (cited above); Yarmush et al (cited above); or the like. Alternatively, a class-specific reagent comprises a solid phase support, *e.g.* a bead, to which a photosensitizer is covalently or non-covalently attached and an antibody is attached, preferably covalently, either directly or by way of a functionalized polymer, such as amino-dextran, or the like.

#### Exemplary Proteins Forming Detectable Complexes

Assays of the invention may be used to determine whether one or more apoptotic pathways are activated by simultaneously measuring protein-protein complexes. Of particular interest are protein-protein complexes that include, but are not limited to, the proteins of the following Table II. Preferably, the human forms of the following proteins and protein families are intended.

Table II.  
Exemplary Target Proteins

Abbreviation	Comments/NCBI references	Reference
<b>14-3-3 protein</b>	BC003047	Aitken et al, Biochem. Soc. Trans., 30: 351-360 (2002); Subramanian et al, Exp. Cell Res., 271: 142-151 (2001)
<b>BAD</b>	BC001901 AF031523 BT006678	Otilie et al, J. Biol. Chem., 272: 30866-30872 (1997); Schurmann et al, Mol. Cell. Biol. 20: 453-461 (2000); Baell et al, Biochem. Pharmacology, 64: 851-863 (2002)
<b>BID</b>		Gross et al, Genes & Development, 13: 1899-1911 (1999); Baell et al (cited above)
<b>BIK/NBK</b>		Gross et al (1999, cited above); Baell et al (cited above)
<b>Blk</b>		Gross et al (1999, cited above); Baell et al (cited above)
<b>Hrk</b>		Gross et al (1999, cited above)
<b>BIM/BOD</b>		Gross et al (1999, cited above); Baell et al (cited above)
<b>NIP3</b>		Gross et al (1999, cited above); Baell et al (cited above)
<b>NIX/BNIP3</b>		Gross et al (1999, cited above); Baell et al (cited above)
<b>Noxa</b>		Antonsson, Cell Tissue Res, 306: 347-361 (2001)

<b>PUMA</b>		Antonsson (cited above)
<b>BAX</b>		Antonsson (cited above); Gross et al, Mol. Cell. Biol. 20: 3125-3136 (2000); Baell et al (cited above)
<b>BAK</b>		Gross et al (1999, cited above)
<b>Bcl-X<sub>S</sub></b>		Gross et al (1999, cited above)
<b>BOK/MTD</b>		Gross et al (1999, cited above)
<b>Bcl-G</b>		Puthalakath et al, Cell Death and Differentiation, 9: 505-512 (2002)
<b>Bcl-2</b>	M14745	Srivastava et al, Proc. Natl. Acad. Sci., 96: 3775-3780 (1999); Baell et al (cited above)
<b>Bcl-X<sub>L</sub></b>		Gross et al (1999, cited above)
<b>Bcl-W</b>		Gross et al (1999, cited above)
<b>MCL-1</b>		Gross et al (1999, cited above)
<b>A1/Bfl-1</b>		Gross et al (1999, cited above)
<b>BOO/DIVA</b>		Gross et al (1999, cited above)
<b>NR-13</b>		Gross et al (1999, cited above)
<b>NF-kB</b>		
<b>I-kB</b>		
<b>caspase-9</b>		Creagh et al, Biochemical Society Transactions, 29: 696-702 (2001);
<b>FADD</b>		Creagh et al (cited above)
<b>TRADD</b>		Creagh et al (cited above)
<b>Apaf-1</b>		Creagh et al (cited above)

Table III.  
Exemplary Protein-Protein Complexes

(where "protein 1//protein 2" indicates a complex comprising protein 1 and protein 2)

14-3-3//BAD	BID//BAX	BAX//BAX	Bcl-X <sub>L</sub> //BAD	Bcl-2//BAD
14-3-3//BID	BID//BAK	BAX//Bcl-2	Bcl-X <sub>L</sub> //BIK	Bcl-2//BIK
NF-kB//I-kB	BID//Bcl-2		Bcl-X <sub>L</sub> //BID	Bcl-2//BID
FADD//caspase-9	BID//Bcl-X <sub>L</sub>		Bcl-X <sub>L</sub> //Hrk	Bcl-2//Hrk
TRADD//caspase-9	BID//A1/Bfl-1		Bcl-X <sub>L</sub> //BIM	Bcl-2//BIM
Apaf-1//caspase-9			Bcl-X <sub>L</sub> //Noxa	Bcl-2//Noxa
			Bcl-X <sub>L</sub> //Bmf	Bcl-2//Bmf
			Bcl-X <sub>L</sub> //Puma	Bcl-2//Puma
			Bcl-X <sub>L</sub> //Bcl-G	Bcl-2//Bcl-G
			Bcl-X <sub>L</sub> //NIP3	Bcl-2//NIP3
			Bcl-X <sub>L</sub> //Nix	Bcl-2//Nix

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#### Assay Conditions

The following general discussion of methods and specific conditions and materials are by way of illustration and not limitation. One of ordinary skill in the art will understand how the

methods described herein can be adapted to other applications, particularly with using different samples, cell types and target complexes.

In conducting the methods of the invention, a combination of the assay components is made, including the sample being tested, the binding compounds, and optionally the cleaving probe. Generally, assay components may be combined in any order. In certain applications, however, the order of addition may be relevant. For example, one may wish to monitor competitive binding, such as in a quantitative assay. Or one may wish to monitor the stability of an assembled complex. In such applications, reactions may be assembled in stages, and may require incubations before the complete mixture has been assembled, or before the cleaving reaction is initiated.

The amounts of each reagent are usually determined empirically. The amount of sample used in an assay will be determined by the predicted number of target complexes present and the means of separation and detection used to monitor the signal of the assay. In general, the amounts of the binding compounds and the cleaving probe are provided in molar excess relative to the expected amount of the target molecules in the sample, generally at a molar excess of at least 1.5, more desirably about 10-fold excess, or more. In specific applications, the concentration used may be higher or lower, depending on the affinity of the binding agents and the expected number of target molecules present on a single cell. Where one is determining the effect of a chemical compound on formation of oligomeric cell surface complexes, the compound may be added to the cells prior to, simultaneously with, or after addition of the probes, depending on the effect being monitored.

The assay mixture is combined and incubated under conditions that provide for binding of the probes to the cell surface molecules, usually in an aqueous medium, generally at a physiological pH (comparable to the pH at which the cells are cultures), maintained by a buffer at a concentration in the range of about 10 to 200 mM. Conventional buffers may be used, as well as other conventional additives as necessary, such as salts, growth medium, stabilizers, etc. Physiological and constant temperatures are normally employed. Incubation temperatures normally range from about 4° to 70°C, usually from about 15° to 45°C, more usually 25° to 37°.

After assembly of the assay mixture and incubation to allow the probes to bind to cell surface molecules, the mixture is treated to activate the cleaving agent to cleave the tags from the binding compounds that are within the effective proximity of the cleaving agent, releasing the corresponding tag from the cell surface into solution. The nature of this treatment will depend on the mechanism of action of the cleaving agent. For example, where a photosensitizer is employed as the cleaving agent, activation of cleavage will comprise irradiation of the mixture at the wavelength of light appropriate to the particular sensitizer used.

Following cleavage, the sample is then analyzed to determine the identity of tags that have been released. Where an assay employing a plurality of binding compounds is employed, separation of the released tags will generally precede their detection. The methods for both separation and detection are determined in the process of designing the tags for the assay. A preferred mode of separation employs electrophoresis, in which the various tags are separated based on known differences in their electrophoretic mobilities.

#### Separation of Released Molecular Tags

As mentioned above, molecular tags are designed for separation by a separation technique that can distinguish molecular tags based on one or more physical, chemical, and/or optical characteristics (referred to herein as "separation characteristics"). As also mentioned above, separation techniques that may be used with the various embodiments of the invention include normal phase or reverse phase HPLC, ion exchange HPLC, capillary electrochromatography, mass spectroscopy, gas phase chromatography, and the like. Preferably, the separation technique selected is capable of providing quantitative information as well as qualitative information about the presence or absence of molecular tags (and therefore, corresponding analytes). In one aspect, a liquid phase separation technique is employed so that a solution, e.g. buffer solution, reaction solvent, or the like, containing a mixture of molecular tags is processed to bring about separation of individual kinds of molecular tags. Usually, such separation is accompanied by the differential movement of molecular tags from such a starting mixture along a path until discernable peaks or bands form that correspond to regions of increased concentration of the respective molecular tags. Such a path may be defined by a fluid flow, electric field, magnetic field, or the like. The selection of a particular separation technique depends on several factors including the expense and convenience of using the technique, the resolving power of the technique given the chemical nature of the molecular tags, the number of molecular tags to be separated, the type of detection mode employed, and the like. Preferably, molecular tags are electrophoretically separated to form an electropherogram in which the separated molecular tags are represented by distinct peaks.

Methods for electrophoresis are well known and there is abundant guidance for one of ordinary skill in the art to make design choices for forming and separating particular pluralities of molecular tags. The following are exemplary references on electrophoresis: Krylov et al, Anal. Chem., 72: 111R-128R (2000); P.D. Grossman and J.C. Colburn, Capillary Electrophoresis: Theory and Practice, Academic Press, Inc., NY (1992); U.S. Patents 5,374,527; 5,624,800; 5,552,028; ABI PRISM 377 DNA Sequencer User's Manual, Rev. A, January 1995, Chapter 2 (Applied Biosystems, Foster City, CA); and the like. In one aspect, molecular tags are separated by capillary electrophoresis. Design choices within the purview of those of ordinary skill include

but are not limited to selection of instrumentation from several commercially available models, selection of operating conditions including separation media type and concentration, pH, desired separation time, temperature, voltage, capillary type and dimensions, detection mode, the number of molecular tags to be separated, and the like.

5 In one aspect of the invention, during or after electrophoretic separation, the molecular tags are detected or identified by recording fluorescence signals and migration times (or migration distances) of the separated compounds, or by constructing a chart of relative fluorescent and order of migration of the molecular tags (*e.g.*, as an electropherogram). Preferably, the presence, absence, and/or amounts of molecular tags are measured by using one  
10 or more standards as disclosed by Williams et al, U.S. patent publication 2003/0170734A1, which is incorporated herein by reference. During or after separation, fluorescent molecular tags can be illuminated by standard means, *e.g.* a high intensity mercury vapor lamp, a laser, or the like. Typically, the molecular tags are illuminated by laser light generated by a He-Ne gas laser or a solid-state diode laser. The fluorescence signals can then be detected by a light-sensitive  
15 detector, *e.g.*, a photomultiplier tube, a charged-coupled device, or the like. Exemplary electrophoresis detection systems are described elsewhere, *e.g.*, U.S. Patent Nos. 5,543,026; 5,274,240; 4,879,012; 5,091,652; 6,142,162; or the like. In another aspect, molecular tags may be detected electrochemically detected, *e.g.* as described in U.S. Patent No. 6,045,676.

Electrophoretic separation involves the migration and separation of molecules in an  
20 electric field based on differences in mobility. Various forms of electrophoretic separation include, by way of example and not limitation, free zone electrophoresis, gel electrophoresis, isoelectric focusing, isotachophoresis, capillary electrochromatography, and micellar electrokinetic chromatography. Capillary electrophoresis involves electroseparation, preferably by electrokinetic flow, including electrophoretic, dielectrophoretic and/or electroosmotic flow,  
25 conducted in a tube or channel of from about 1 to about 200 micrometers, usually, from about 10 to about 100 micrometers cross-sectional dimensions. The capillary may be a long independent capillary tube or a channel in a wafer or film comprised of silicon, quartz, glass or plastic.

In capillary electroseparation, an aliquot of the reaction mixture containing the molecular tags is subjected to electroseparation by introducing the aliquot into an electroseparation channel  
30 that may be part of, or linked to, a capillary device in which the amplification and other reactions are performed. An electric potential is then applied to the electrically conductive medium contained within the channel to effectuate migration of the components within the combination. Generally, the electric potential applied is sufficient to achieve electroseparation of the desired components according to practices well known in the art. One skilled in the art will be capable  
35 of determining the suitable electric potentials for a given set of reagents used in the present invention and/or the nature of the cleaved labels, the nature of the reaction medium and so forth.

The parameters for the electroseparation including those for the medium and the electric potential are usually optimized to achieve maximum separation of the desired components. This may be achieved empirically and is well within the purview of the skilled artisan. Detection may be by any of the known methods associated with the analysis of capillary electrophoresis columns including the methods shown in U.S. Patent Nos. 5,560,811 (column 11, lines 19-30), 4,675,300, 4,274,240 and 5,324,401, the relevant disclosures of which are incorporated herein by reference. Those skilled in the electrophoresis arts will recognize a wide range of electric potentials or field strengths may be used, for example, fields of 10 to 1000 V/cm are used with about 200 to about 600 V/cm being more typical. The upper voltage limit for commercial systems is about 30 kV, with a capillary length of about 40 to about 60 cm, giving a maximum field of about 600 V/cm. For DNA, typically the capillary is coated to reduce electroosmotic flow, and the injection end of the capillary is maintained at a negative potential.

For ease of detection, the entire apparatus may be fabricated from a plastic material that is optically transparent, which generally allows light of wavelengths ranging from about 180 to about 1500 nm, usually about 220 to about 800 nm, more usually about 450 to about 700 nm, to have low transmission losses. Suitable materials include fused silica, plastics, quartz, glass, and so forth.

In one aspect of the invention, molecular tags are separated by electrophoresis in a microfluidics device, as illustrated diagrammatically in Figs. 6A-6C. Microfluidics devices are described, for example, in U.S. Patent nos. 5,750,015; 5,900,130; 6,007,690; and WO 98/45693; WO 99/19717 and WO 99/15876. Conveniently, an aliquot, generally not more than about 5  $\mu$ l, is transferred to the sample reservoir of a microfluidics device, either directly through electrophoretic or pneumatic injection into an integrated system or by syringe, capillary or the like. The conditions under which the separation is performed are conventional and will vary with the nature of the products.

By way of illustration, Figs. 6A-6C show a microchannel network 100 in a microfluidics device of the type detailed in the application noted above, for sample loading and electrophoretic separation of a sample of probes and tags produced in the assay above. Briefly, the network includes a main separation channel 102 terminating at upstream and downstream reservoirs 104, 106, respectively. The main channel is intersected at offset axial positions by a side channel 108 that terminates at a reservoir 110, and a side channel 112 that terminates at a reservoir 114. The offset between the two-side channels forms a sample loading zone 116 within the main channel.

In operation, an assay mixture is placed in sample reservoir 110, illustrated in Fig. 6A. As noted, the assay mixture contains one or more target cells with surface-bound cleaving agent, one or more protein probes, and optionally, molecular tag standard. The assay reaction, involving initial probe binding to target cell(s), followed by cleavage of probe linkers in probe-

bound cells, may be carried out in sample reservoir 110, or alternatively, the assay reactions can be carried out in another reaction vessel, with the reacted sample components the added to the sample reservoir.

To load released molecular tags into the sample-loading zone, an electric field is applied across reservoirs 110, 114, in the direction indicated in Fig. 6B, wherein negatively charged released molecular tags are drawn from reservoir 110 into loading zone 116, while uncharged or positively charged sample components remain in the sample reservoir. The released tags in the loading zone can now be separated by conventional capillary electrophoresis, by applying an electric field across reservoirs 104, 106, in the direction indicated in Fig. 6C.

From the resulting electrophoretic pattern, the molecular tags, and corresponding analytes, can be identified. This is typically done by placing a fluorescence detector near the downstream end of the separation channel, and constructing an electropherogram of the separated molecular tags, first to determine the separation characteristic (in this case, electrophoretic mobility) as above, and secondly, to measure signal intensity, *e.g.*, peak height or peak area, as a measure of the relative amount of tag associated with each probe. Methods for detecting and quantifying levels of a detectable probe are well known. In one preferred method, the molecular tags are fluorescently labeled. A standard fluorescence-emission source is directed against a detection zone in a downstream portion of the separation medium, and fluorescence emission of the zone is measured by a standard light detector. The signal height or area recorded provides a measure of product and substrate concentration in the sample. With the above detection information, it is now possible to assign each detected molecular tag to a particular probe in the probe set, and to compare the relative levels of each detectable probe, as a measure of its relatively substrate conversion or ligand binding.

In one aspect of the invention, pluralities of molecular tags are designed for separation by chromatography based on one or more physical characteristics that include but are not limited to molecular weight, shape, solubility, pKa, hydrophobicity, charge, polarity, or the like. A chromatographic separation technique is selected based on parameters such as column type, solid phase, mobile phase, and the like, followed by selection of a plurality of molecular tags that may be separated to form distinct peaks or bands in a single operation. Several factors determine which HPLC technique is selected for use in the invention, including the number of molecular tags to be detected (*i.e.* the size of the plurality), the estimated quantities of each molecular tag that will be generated in the assays, the availability and ease of synthesizing molecular tags that are candidates for a set to be used in multiplexed assays, the detection modality employed, and the availability, robustness, cost, and ease of operation of HPLC instrumentation, columns, and solvents. Generally, columns and techniques are favored that are suitable for analyzing limited amounts of sample and that provide the highest resolution separations. Guidance for making

such selections can be found in the literature, e.g. Snyder et al, Practical HPLC Method Development, (John Wiley & Sons, New York, 1988); Millner, "High Resolution Chromatography: A Practical Approach", Oxford University Press, New York (1999), Chi-San Wu, "Column Handbook for Size Exclusion Chromatography", Academic Press, San Diego (1999), and Oliver, "HPLC of Macromolecules: A Practical Approach, Oxford University Press", Oxford, England (1989). In particular, procedures are available for systematic development and optimization of chromatographic separations given conditions, such as column type, solid phase, and the like, e.g. Haber et al, J. Chromatogr. Sci., 38: 386-392 (2000); Outinen et al, Eur. J. Pharm. Sci., 6: 197-205 (1998); Lewis et al, J. Chromatogr., 592: 183-195 and 197-208 (1992); and the like.

In one aspect, initial selections of molecular tag candidates are governed by the physiochemical properties of molecules typically separated by the selected column and stationary phase. The initial selections are then improved empirically by following conventional optimization procedure, as described in the above reference, and by substituting more suitable candidate molecular tags for the separation objectives of a particular embodiment. In one aspect, separation objectives of the invention include (i) separation of the molecular tags of a plurality into distinguishable peaks or bands in a separation time of less than 60 minutes, and more preferably in less than 40 minutes, and still more preferably in a range of between 10 to 40 minutes, (ii) the formation of peaks or bands such that any pair has a resolution of at least 1.0, more preferably at least 1.25, and still more preferably, at least 1.50, (iii) column pressure during separation of less than 150 bar, (iv) separation temperature in the range of from 25°C to 90°C, preferably in the range of from 35°C to 80°C, and (v) the plurality of distinguishable peaks is in the range of from 5 to 30 and all of the peaks in the same chromatogram. As used herein, "resolution" in reference to two peaks or bands is the distance between the two peak or band centers divided by the average base width of the peaks, e.g. Snyder et al (cited above). A variety of commercially available systems are well-suited for high throughput chromatographic analysis of molecular tags. Those skilled in the art can determine appropriate equipment, such as automated sample preparation systems and autoinjection systems, useful for automating HPLC analysis of molecular tags. Automated methods can be used for high-throughput analysis of molecular tags, for example, when a large number of samples are being processed or for multiplexed application of the methods of the invention for detecting target analytes. An exemplary HPLC instrumentation system suitable for use with the present invention is the Agilent 1100 Series HPLC system (Agilent Technologies, Palo Alto, CA).

#### Synthesis of Assay Reagents

Binding compounds for use of the invention are synthesized as disclosed in the following references, which are incorporated herein by reference: International patent publications WO 00/66607; WO 01/83502; WO 02/95356; WO 03/06947; and U.S. patents 6,322,980 and 6,514,700. Exemplary reagents for synthesis of binding compounds are shown in Figures 4 A-J.

5 Exemplary synthesis protocols are illustrated in Figures 5A-7D.

### Example

#### Simultaneous Measurement of 14-3-3//BAD and Bcl-2//BAD Apoptotic Complexes

10 In this example, an assay, illustrated in Figs. 7A-7C, is described for measuring relative amounts of two complexes that contain BAD protein: (i) 14-3-3 protein//BAD protein, and (ii) Bcl-2 protein//BAD protein. Cleaving probe (706) is specific for BAD protein (700) at a first antigenic determinant, binding compound (708) is specific for BAD protein (700) at a second antigenic determinant, binding compound (710) is specific for 14-3-3 protein (702), and binding  
15 compound (712) is specific for Bcl-2 protein (704). The cleaving probe and binding compounds are selected so that their binding sites do not interfere with one another and that site are not selected that are hidden when either complex forms. The assays are carried out as follows.

#### Sample Preparation:

- 20 1. Serum-starve breast cancer cell line culture (MCF-7) overnight before use.
2. Stimulate cell lines with HRG in culture media for 10 minutes at 37°C. Exemplary doses of HRG are 0, 0.032, 0.16, 0.8, 4, 20, 100 nM for MCF-7 cells.
3. Aspirate culture media, transfer onto ice, and add lysis buffer (described below) to lyse cells in situ.
- 25 4. Scrape and transfer lysate to microfuge tube. Incubate on ice for 30 min. Microfuge at 14,000 rpm, 4°C, for 10 min.
5. Collect supernatants as lysates and aliquot for storage at -80°C until use.

#### Lysis Buffer (made fresh and stored on ice):

30 <u>Final</u>	<u>ul</u>	<u>Stock</u>
1% Triton X-100	1000	10%
20 mM Tris-HCl (pH 7.5)	200	1 M
100 mM NaCl	200	5 M
50 mM NaF	500	1 M
35 50 mM Na beta-glycerophosphate	1000	0.5 M
1 mM Na <sub>3</sub> VO <sub>4</sub>	100	0.1 M

5 mM EDTA	100	0.5 M
10 ug/ml pepstatin	100	1 mg/ml
1 tablet (per 10 ml) Roche Complete protease inhibitor (#1836170)	N/A	N/A
Water	<u>6500</u>	<u>N/A</u>
5	10 ml Total	

The total assay volume is 40 ul. The lysate volume is adjusted to 10 ul with lysis buffer. The antibodies are diluted in lysis buffer up to 20 ul. Typically ~5000 to 500,000 cell-equivalent of lysates is used per reaction.

- 10 Procedure: Working concentrations of pre-mixed antibodies prior to adding into reaction:  
For Her-3/PI3K complex with cleaving probe at Her-3 (the design of Fig. 7A)

eTag1\_anti-BAD at 10 nM

eTag2\_anti-Bcl-2 at 10 nM

- 15 eTag3\_anti-14-3-3 at 10 nM

Biotin\_anti-BAD at 20 nM

Universal Standard US-1 at 700 nM

[The Universal Standard US-1 is BSA conjugated with biotin and molecular tag Pro8, which is used to normalize the amount of streptavidin-photosensitizer beads in an assay]. The

- 20 molecular tags are attached directly to antibodies by reacting an NHS-ester of a molecular tag precursor (see Figs. 4A-4J) with free amines on the antibodies using conventional techniques, e.g. Hermanson (cited above).

- 25 1. To assay 96-well filter plate (Millipore MAGVN2250), add 20 ul antibody mix to 10 ul lysate and incubate for 1 hour at 4°C.
2. Add 10 ul streptavidin-derivatized cleaving probe (final 4 ug/well) to assay well and incubate for 40 min.
3. Add 200 ul wash buffer and apply vacuum to empty.
4. Add 30 ul illumination buffer and illuminate.
- 30 5. Transfer 10 ul of each reaction to CE assay plate for analysis.

#### Data Analysis:

- 35 1. Normalize relative fluorescence units (RFU) signal of each molecular tag against that of internal Universal Standard US-1.

2. Subtract RFU of “no lysate” background control from corresponding normalized eTag reporter signals.

The assay readout may be provided as bar graphs as illustrated in Figs. 7B and 7C. These figures  
5 illustrate how the distribution of BAD between 14-3-3 and Bcl-2 would be manifested. In Figs.  
7B and 7C, the leftmost bar for molecular tag 1, “mT<sub>1</sub>,” is a measure of the total amount of BAD  
in the assay, either in complex with 14-3-3, in complex with Bcl-2, or in monomeric form. In  
Fig. 7B, a situation is illustrated in which relatively more BAD is bound to 14-3-3 than Bcl-2, as  
indicated by the rightmost bar being larger than the middle bar. In Fig. 7C, the converse  
10 situation is illustrated; namely, relatively more BAD is bound to Bcl-2 than 14-3-3, as indicated  
by the rightmost bar being smaller than the middle bar.

What is claimed is:

1. A method of determining an apoptotic status of cells in a sample, the method comprising the step of simultaneously measuring amounts of at least one complex selected from the group consisting of a complex comprising a Bcl-2 protein and a BH3-only protein and a complex comprising a 14-3-3 protein and a BAD protein.

2. The method of claim 1 further including simultaneously measuring free NF-kB proteins and free I-kB proteins, and wherein said group further consist of a complex comprising an NF-kB protein and an I-kB protein.

3. The method of claim 2 wherein said group further consists of a homodimer comprising a BAX protein.

4. A method of determining a status of a cancer in a patient, the method comprising the step of simultaneously measuring in a sample from the patient amounts of at least one complex selected from the group consisting of a complex comprising a Bcl-2 protein and a BH3-only protein and a complex comprising a 14-3-3 protein and a BAD protein.

5. A method of determining an apoptotic status of cells in a sample, the method comprising the step of simultaneously measuring amounts of at least one complex selected from the group consisting of a complex comprising an NF-kB protein and an I-kB protein and amounts of free NF-kB proteins and free I-kB proteins.

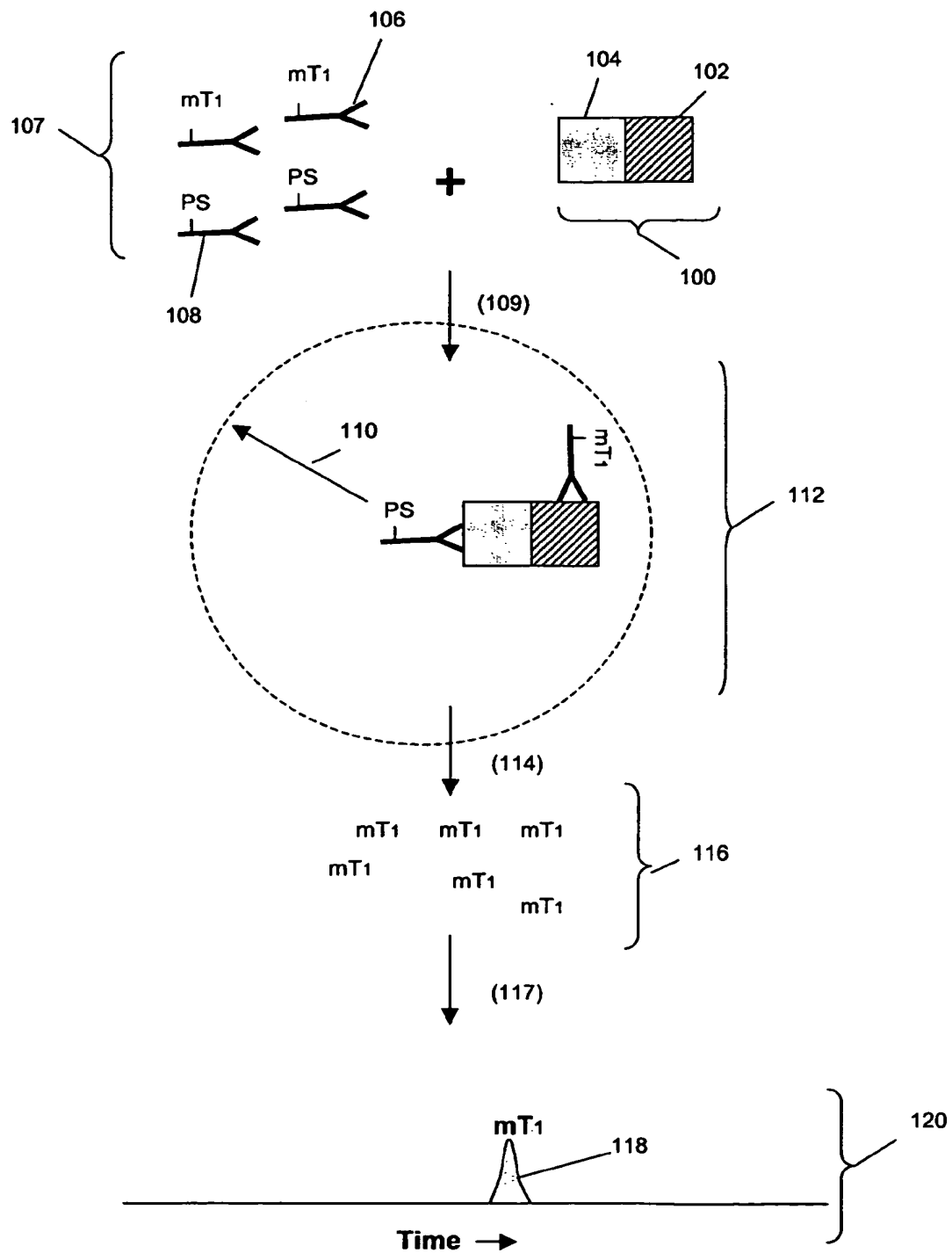
## **DETERMINING APOPTOTIC STATUS IN NEOPLASTIC DISORDERS**

5

### **Abstract of the Invention**

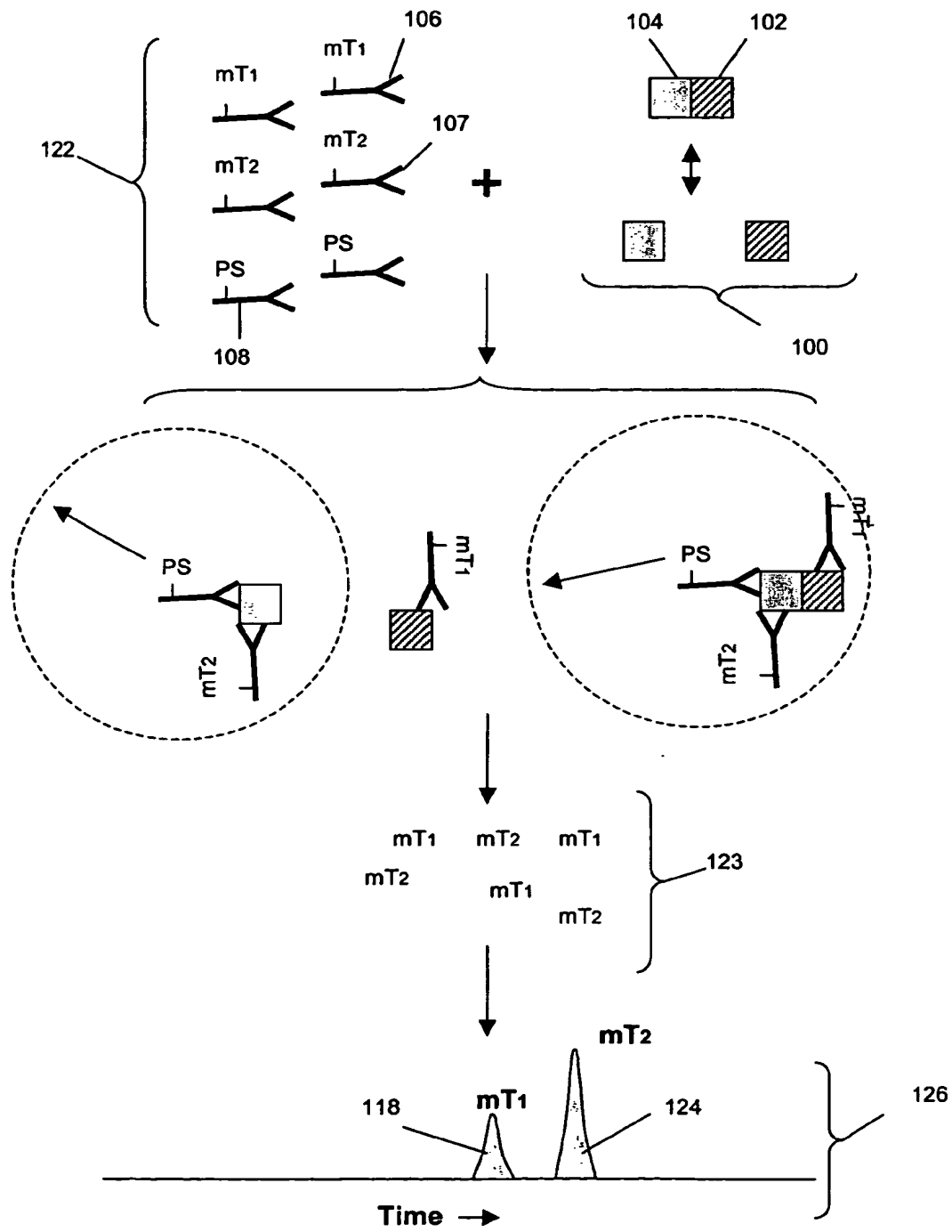
10 The invention provides a method for determining the activation status of apoptotic pathways in either cell samples or patient samples by measuring relative amounts of protein-protein complexes that are characteristic of the apoptotic pathways. In particular, the invention provides a method of determining the activation status of the mitochondrial apoptotic pathway by simultaneously measuring relative amounts of complexes between 14-3-3 proteins and BAD proteins on the one hand and complexes of Bcl-2 proteins and BAD proteins on the other hand.

15 Preferably, methods of the invention are implemented by using sets of binding compounds having releasable molecular tags that are specific for multiple components of one or more complexes formed within apoptotic pathways. After binding, molecular tags are released and separated from the assay mixture for analysis.



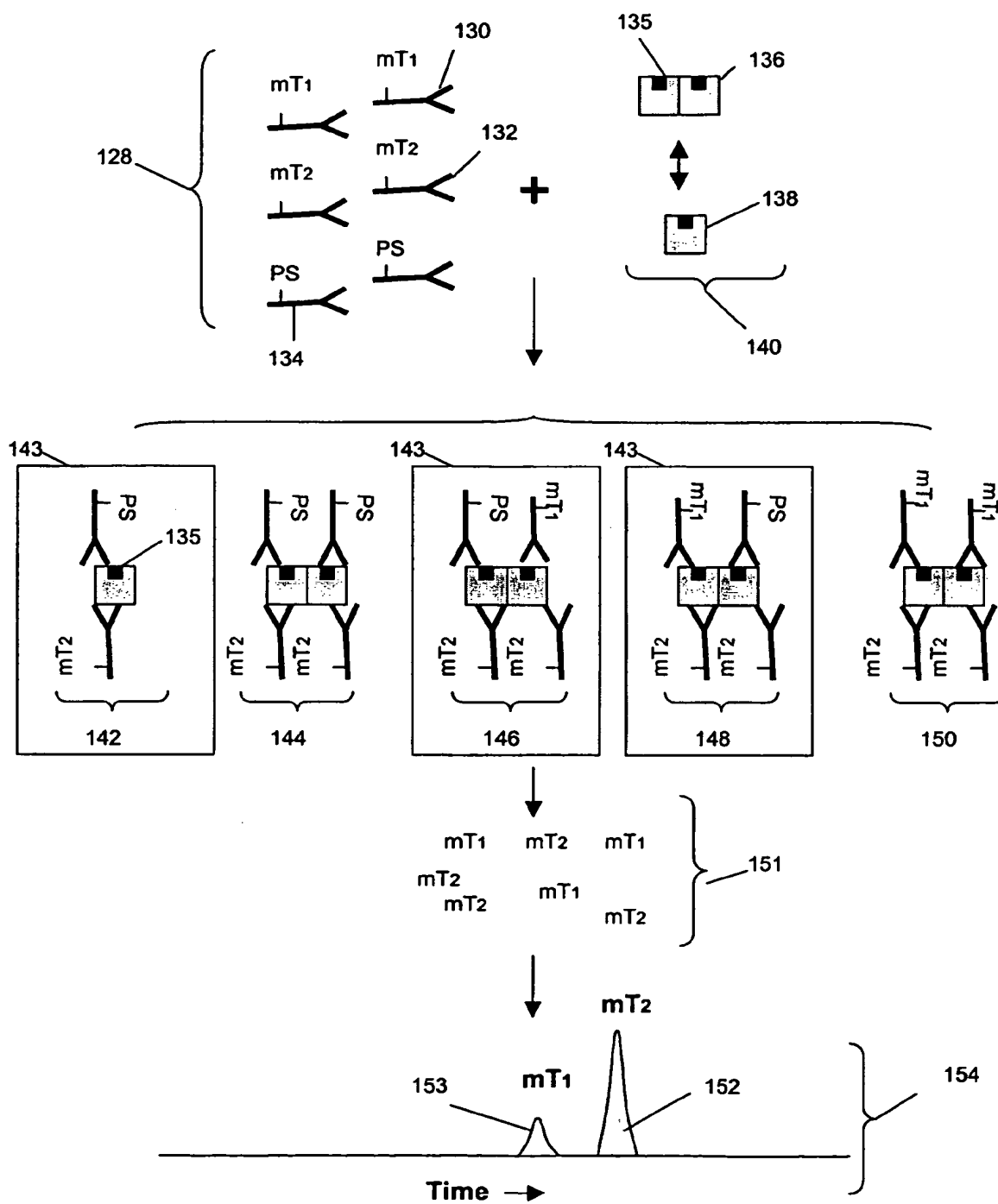
**Fig. 1A**

# Heterodimers

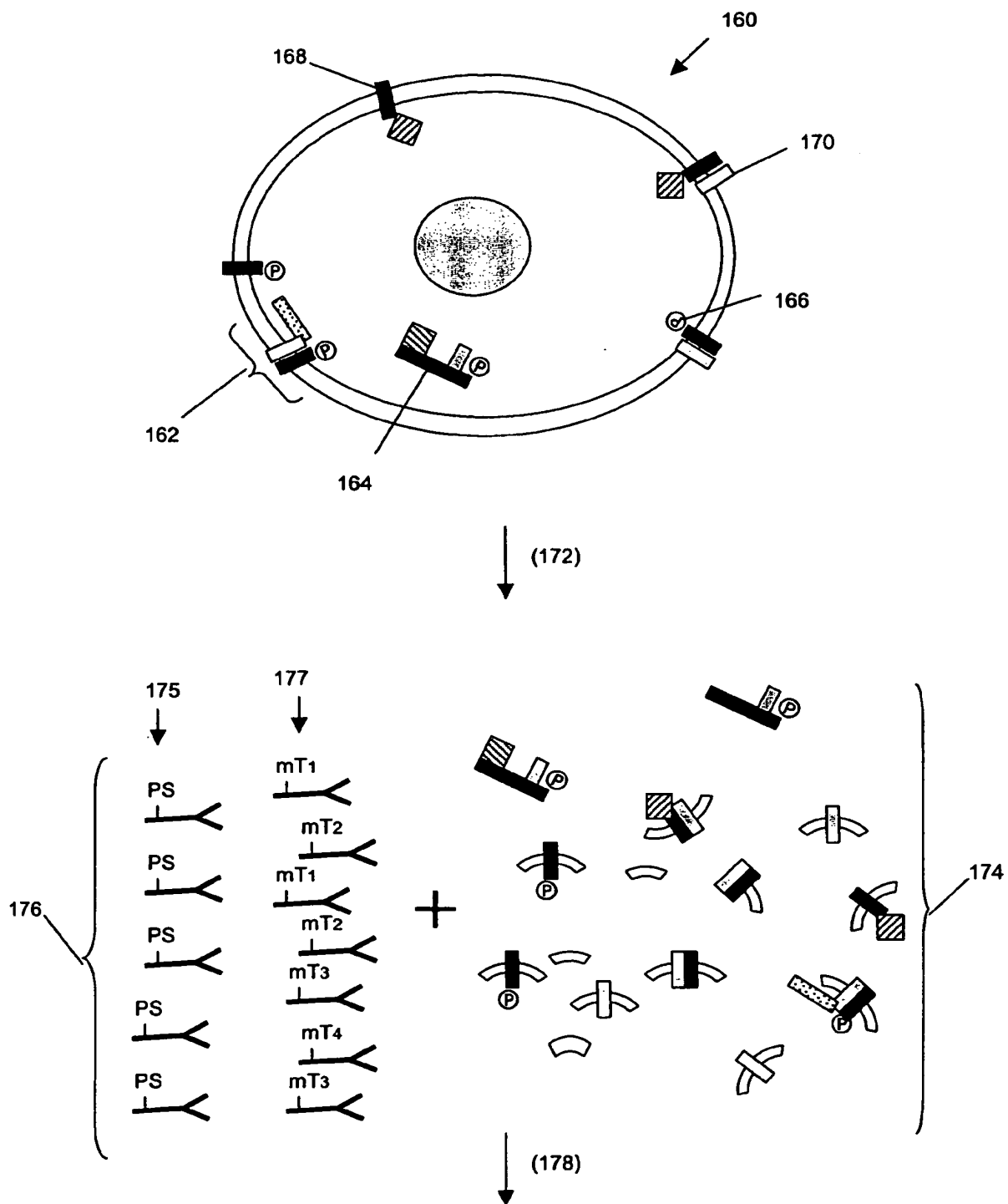


**Fig. 1B**

# Homodimers

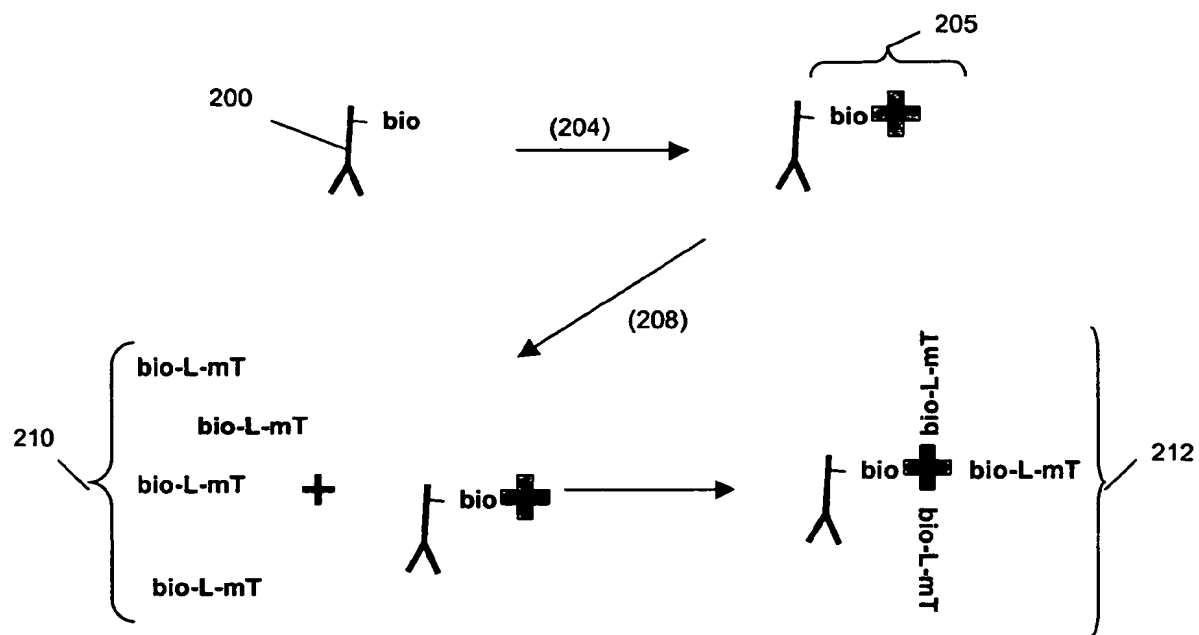


**Fig. 1C**

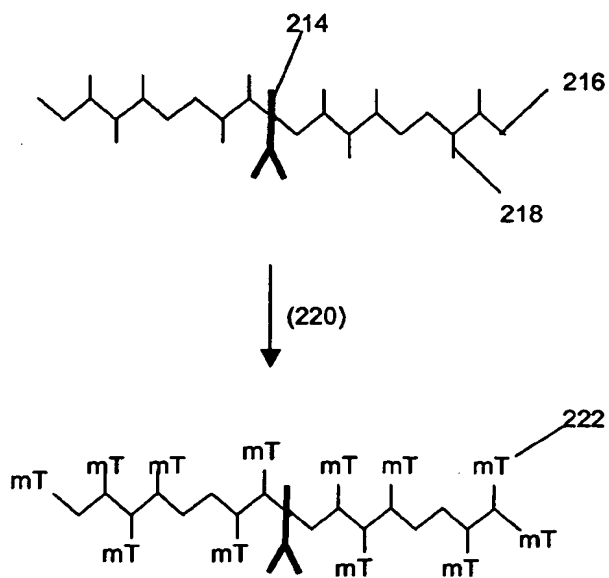


**Fig. 1D**



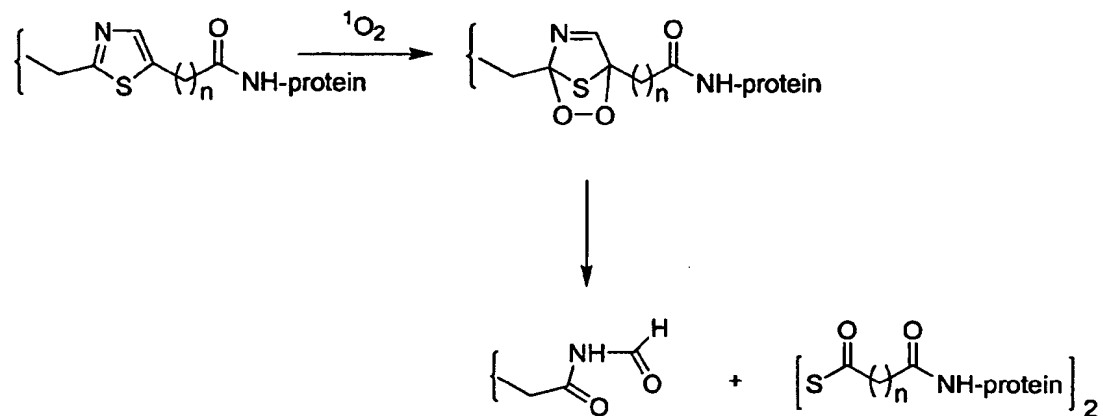


**Fig. 2A**



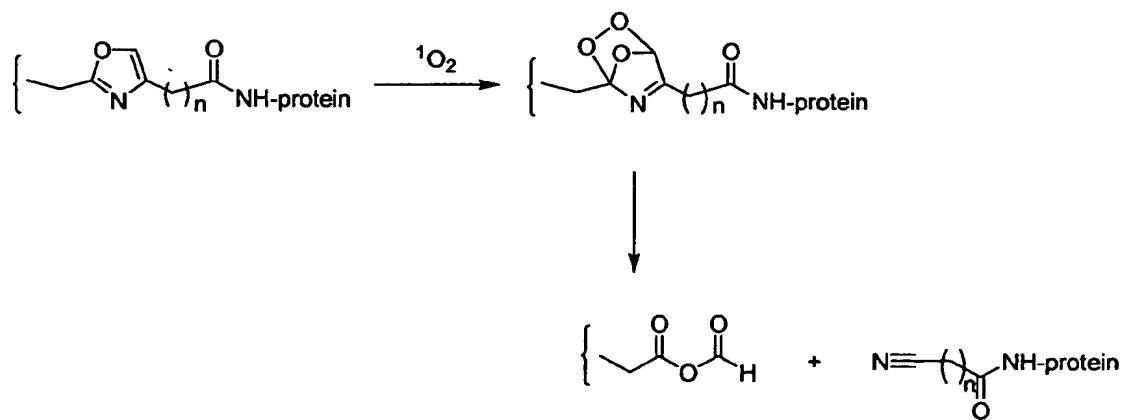
**Fig. 2B**

**Thiazole cleavable linkage**



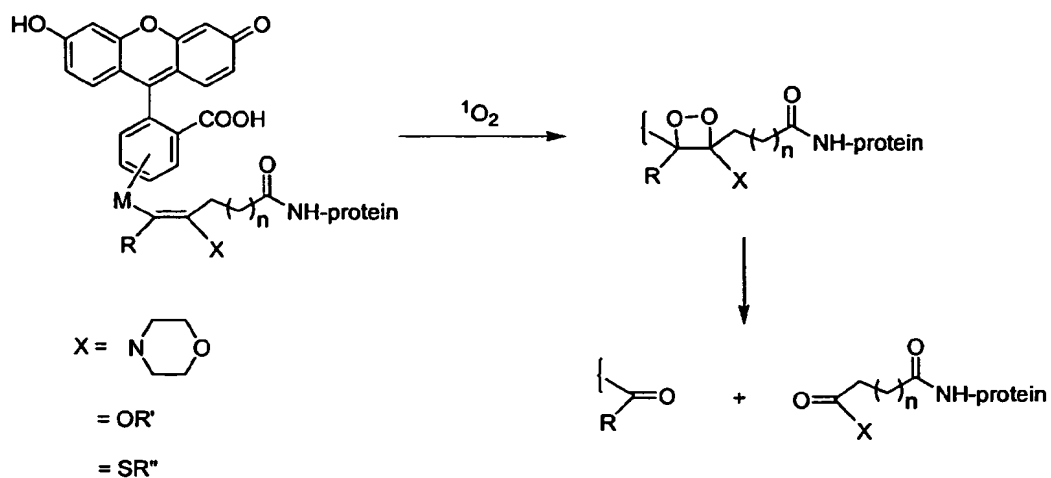
**Fig. 3A**

**Oxazole cleavable linkage**



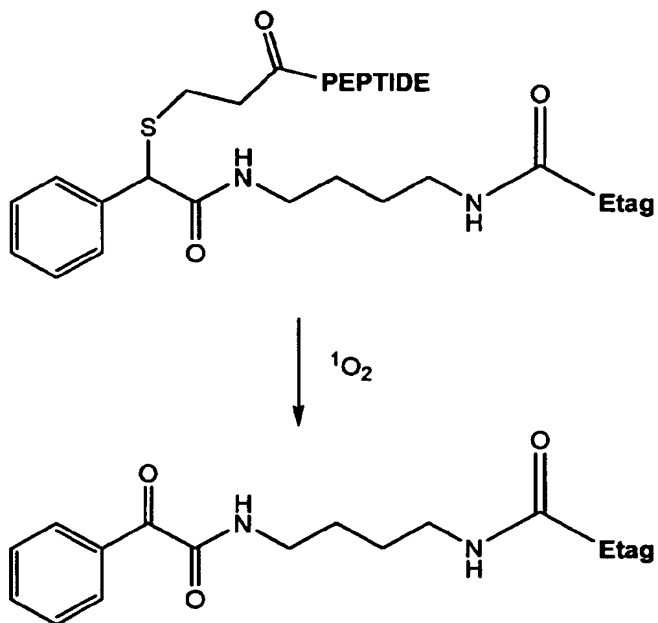
**Fig. 3B**

**Olefin cleavable linkage**

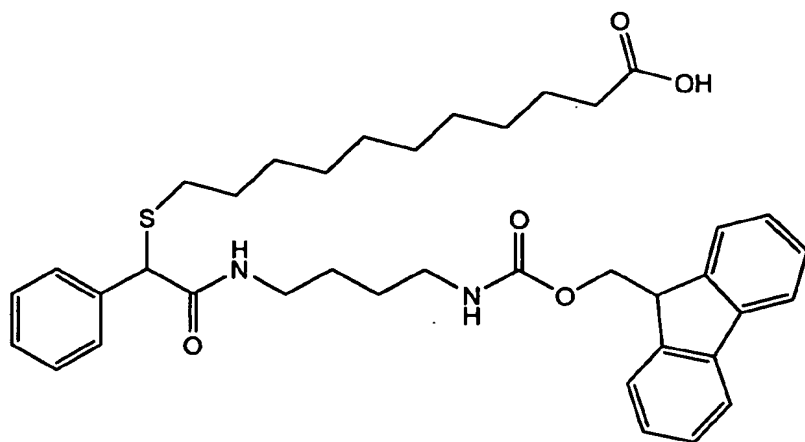


**Fig. 3C**

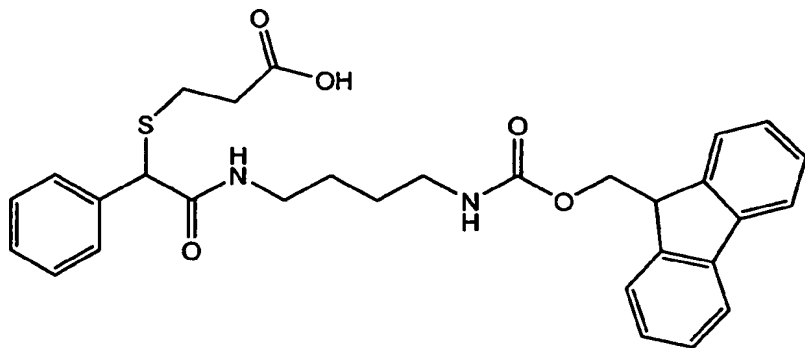
**Thioether cleavable linkage**



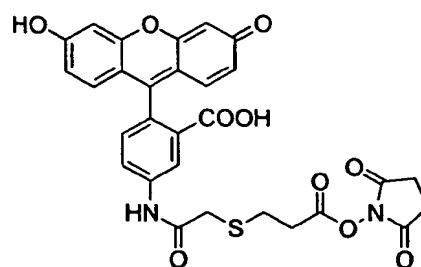
**Fig. 3D**



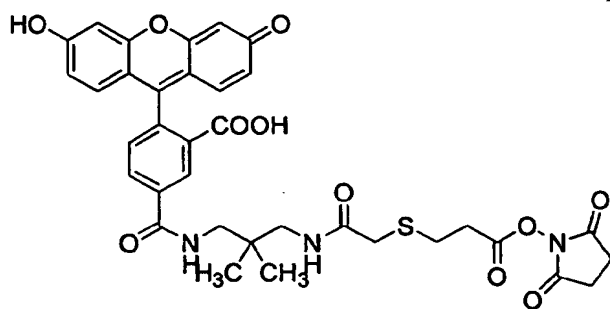
**Fig. 3E**



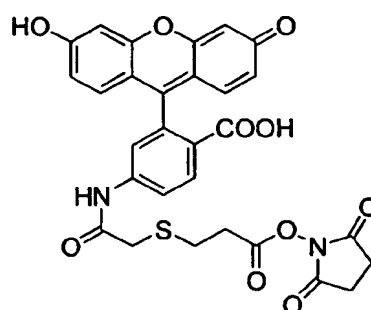
**Fig. 3F**



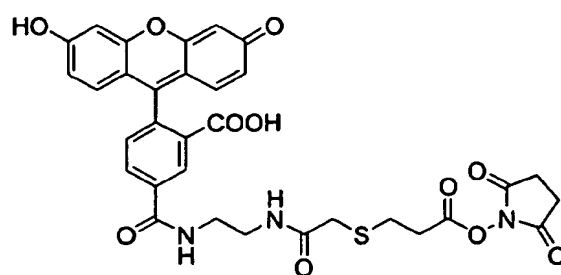
**Pro1-NHS**



**Pro2-NHS**

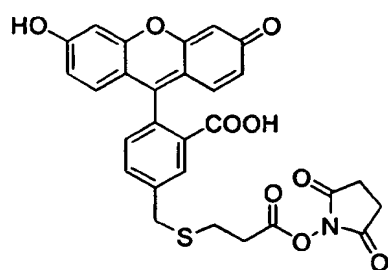


**Pro3-NHS**

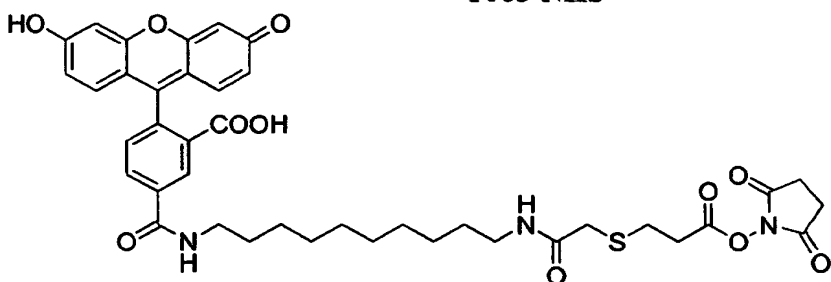


**Pro4-NHS**

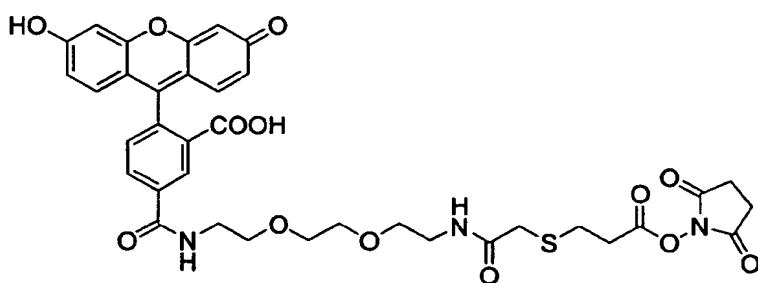
**Fig. 4A**



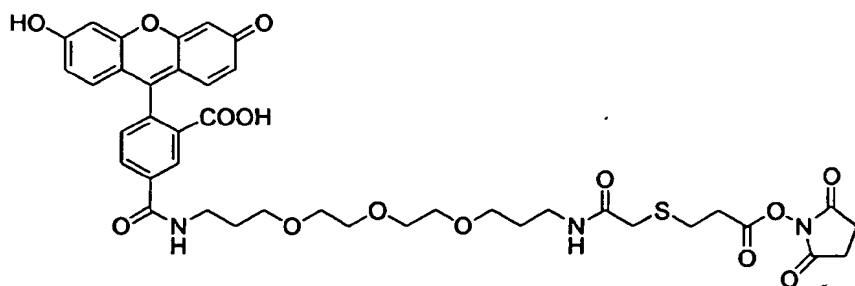
**Pro5-NHS**



**Pro6-NHS**

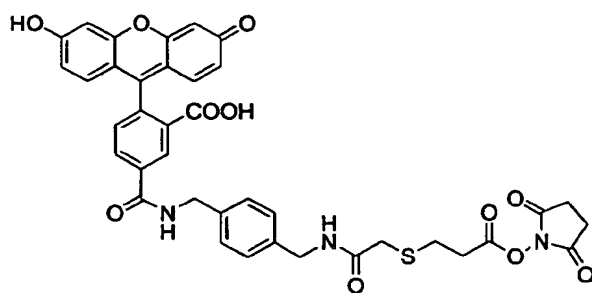


**Pro7-NHS**

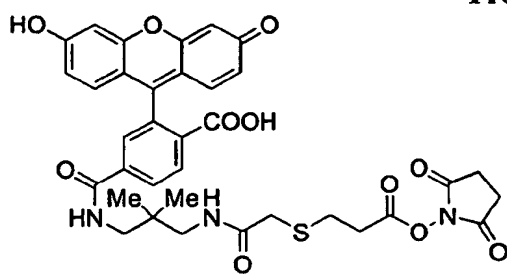


**Pro8-NHS**

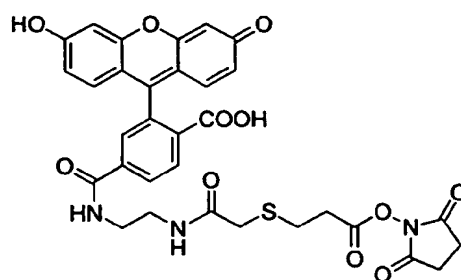
**Fig. 4B**



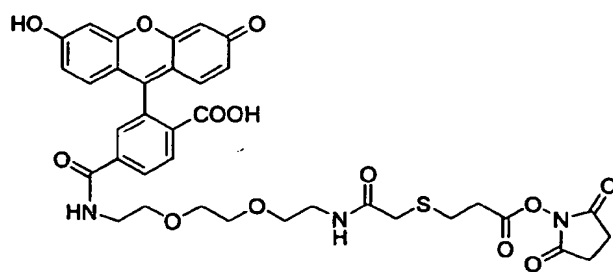
**Pro9-NHS**



**Pro10-NHS**

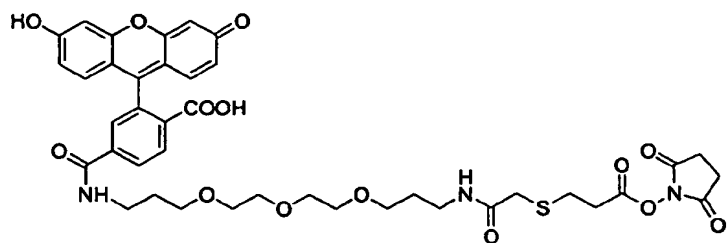


**Pro11-NHS**

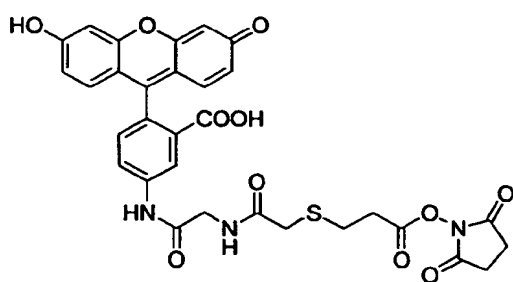


**Pro12-NHS**

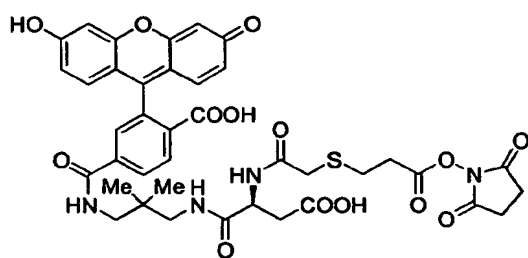
**Fig. 4C**



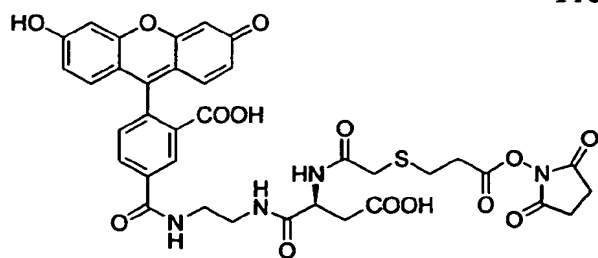
**Pro13-NHS**



**Pro14-NHS**

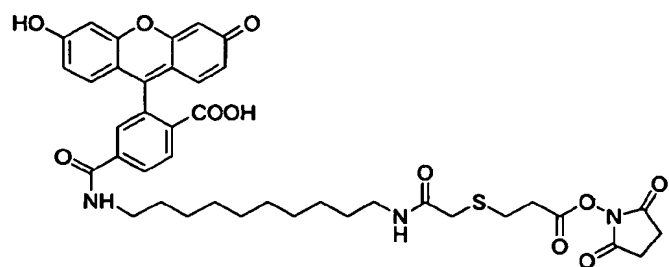


**Pro15-NHS**

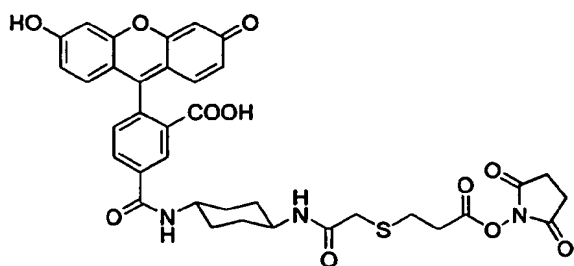


**Pro16-NHS**

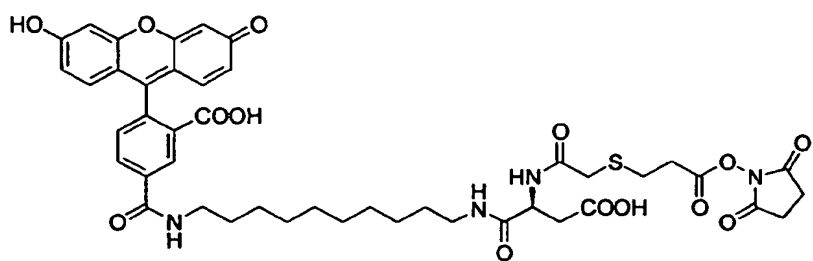
**Fig. 4D**



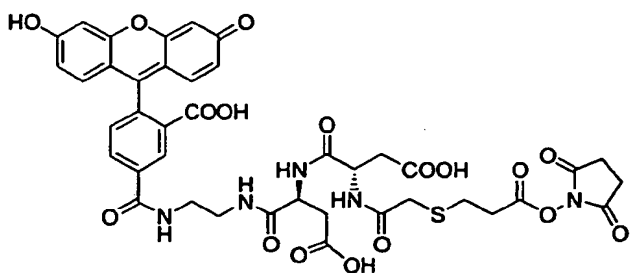
**Pro17-NHS**



**Pro18-NHS**

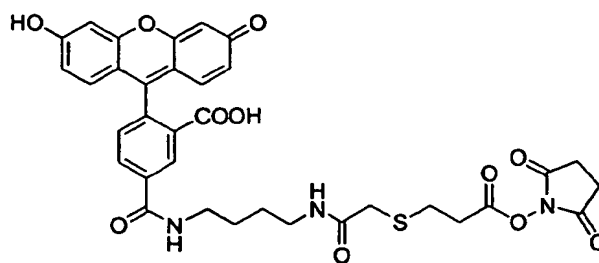


**Pro19-NHS**

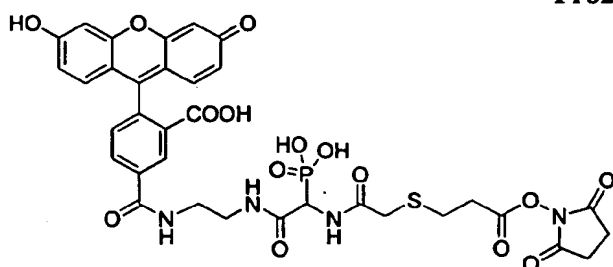


**Pro20-NHS**

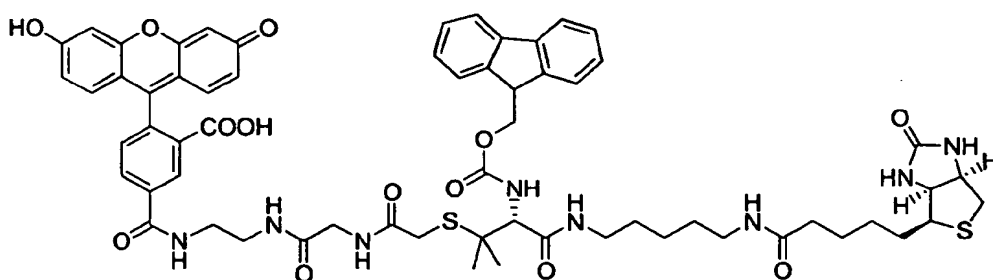
**Fig. 4E**



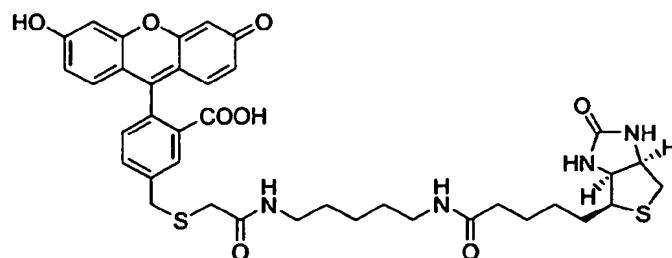
**Pro21-NHS**



**Pro22-NHS**

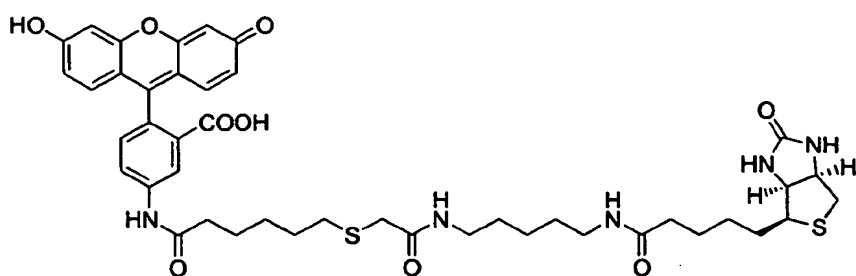


**Pro23-biotin**

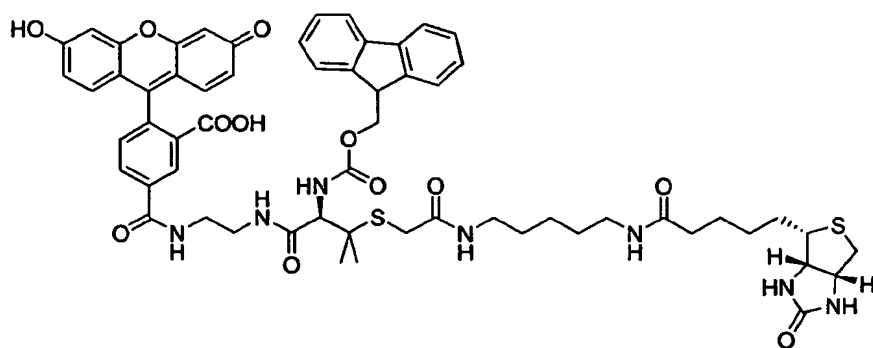


**Pro24-biotin**

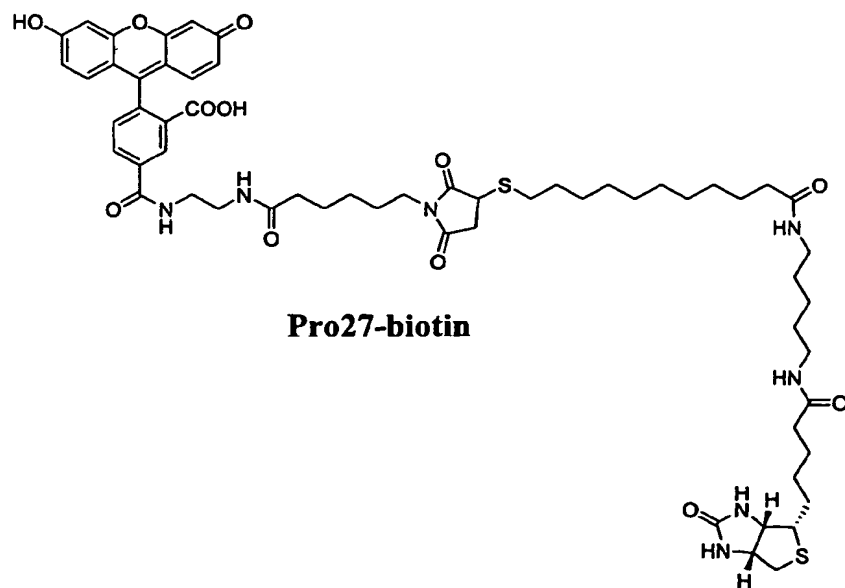
**Fig. 4F**



**Pro25-biotin**

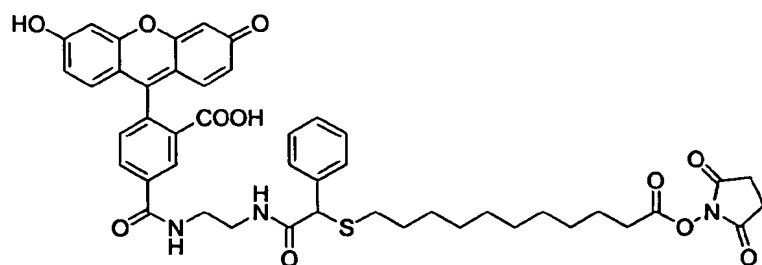


**Pro26-biotin**

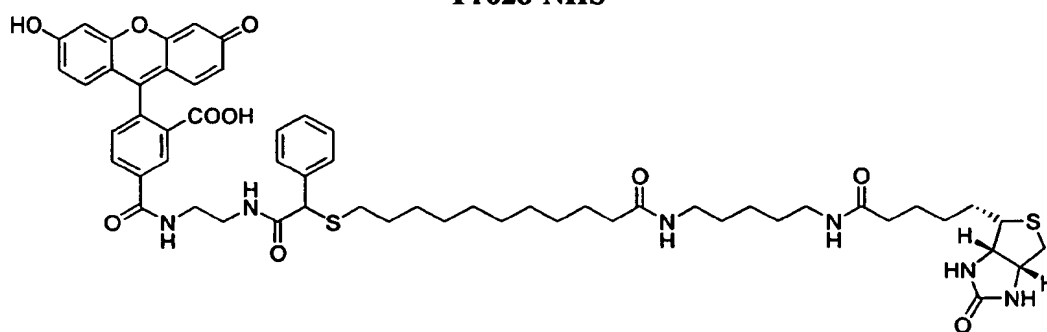


**Pro27-biotin**

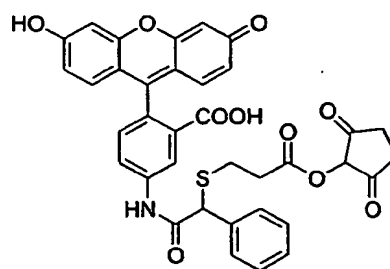
**Fig. 4G**



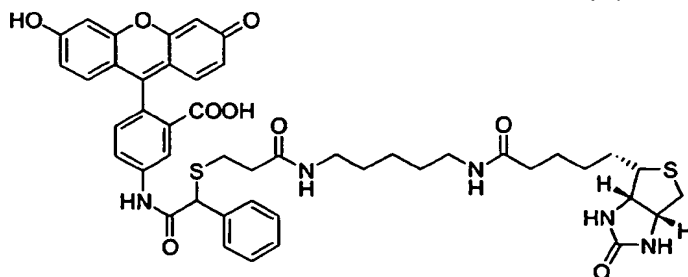
**Pro28-NHS**



**Pro28-biotin**

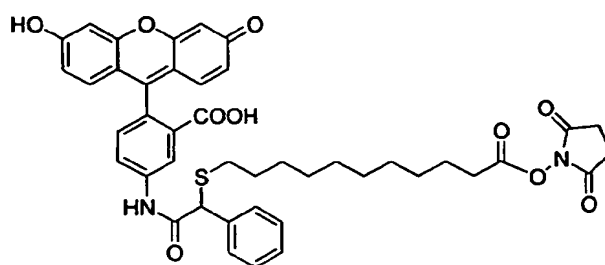


**Pro29-NHS**

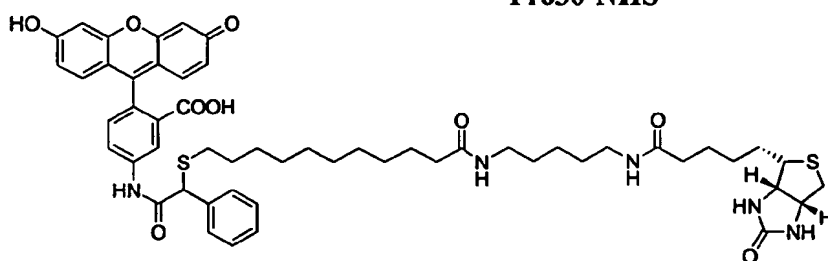


**Pro29-biotin**

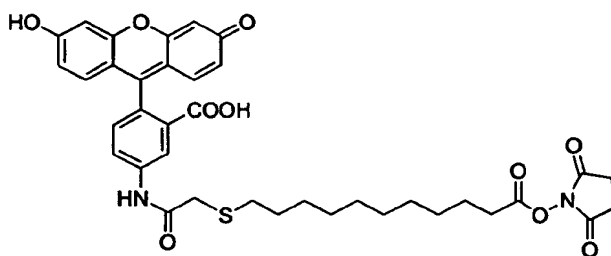
**Fig. 4H**



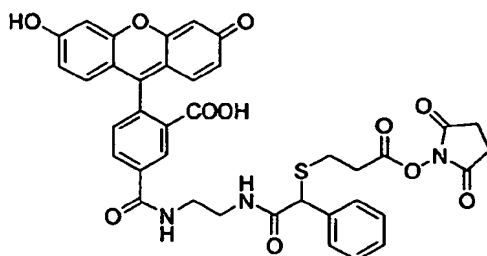
**Pro30-NHS**



**Pro30-biotin**

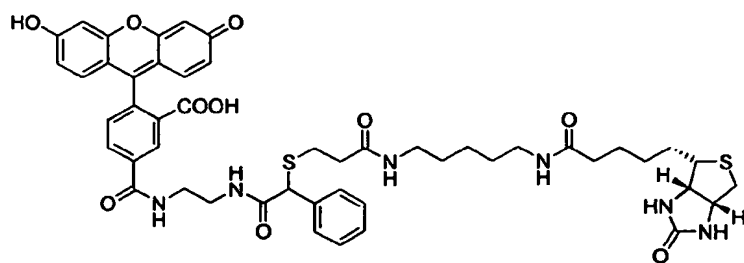


**Pro31-NHS**

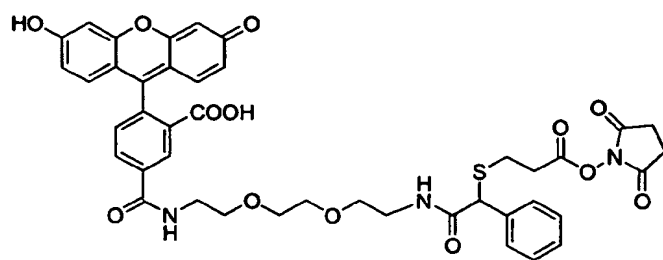


**Pro32-NHS**

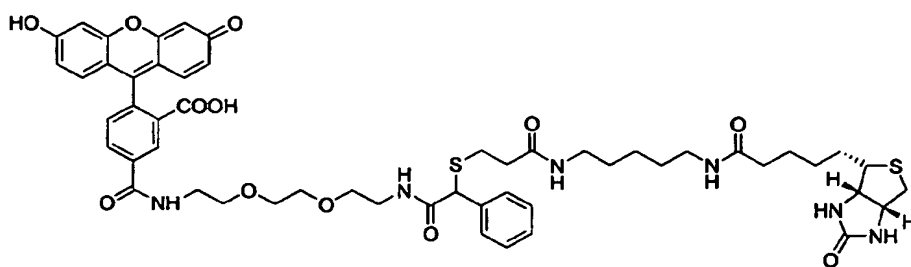
**Fig. 4I**



**Pro32-biotin**



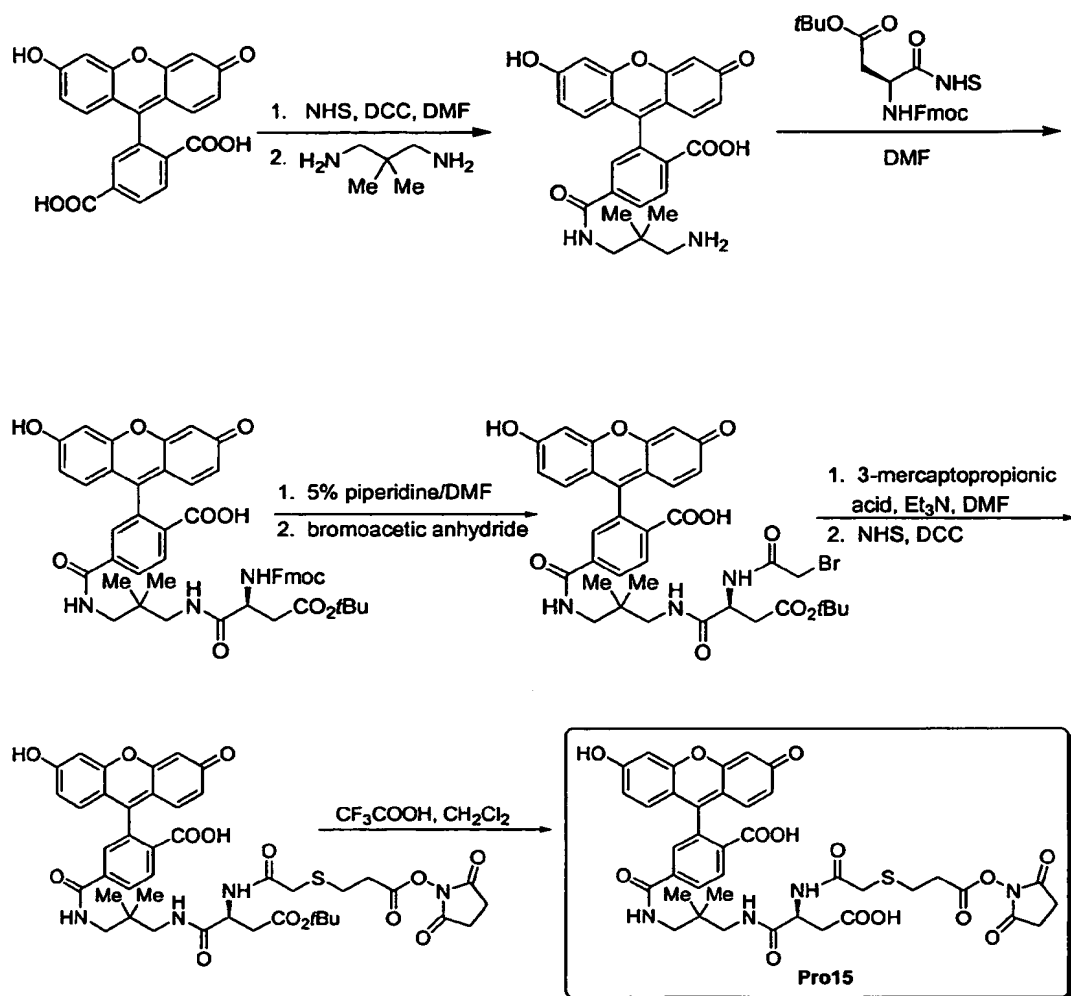
**Pro33-NHS**



**Pro33-biotin**

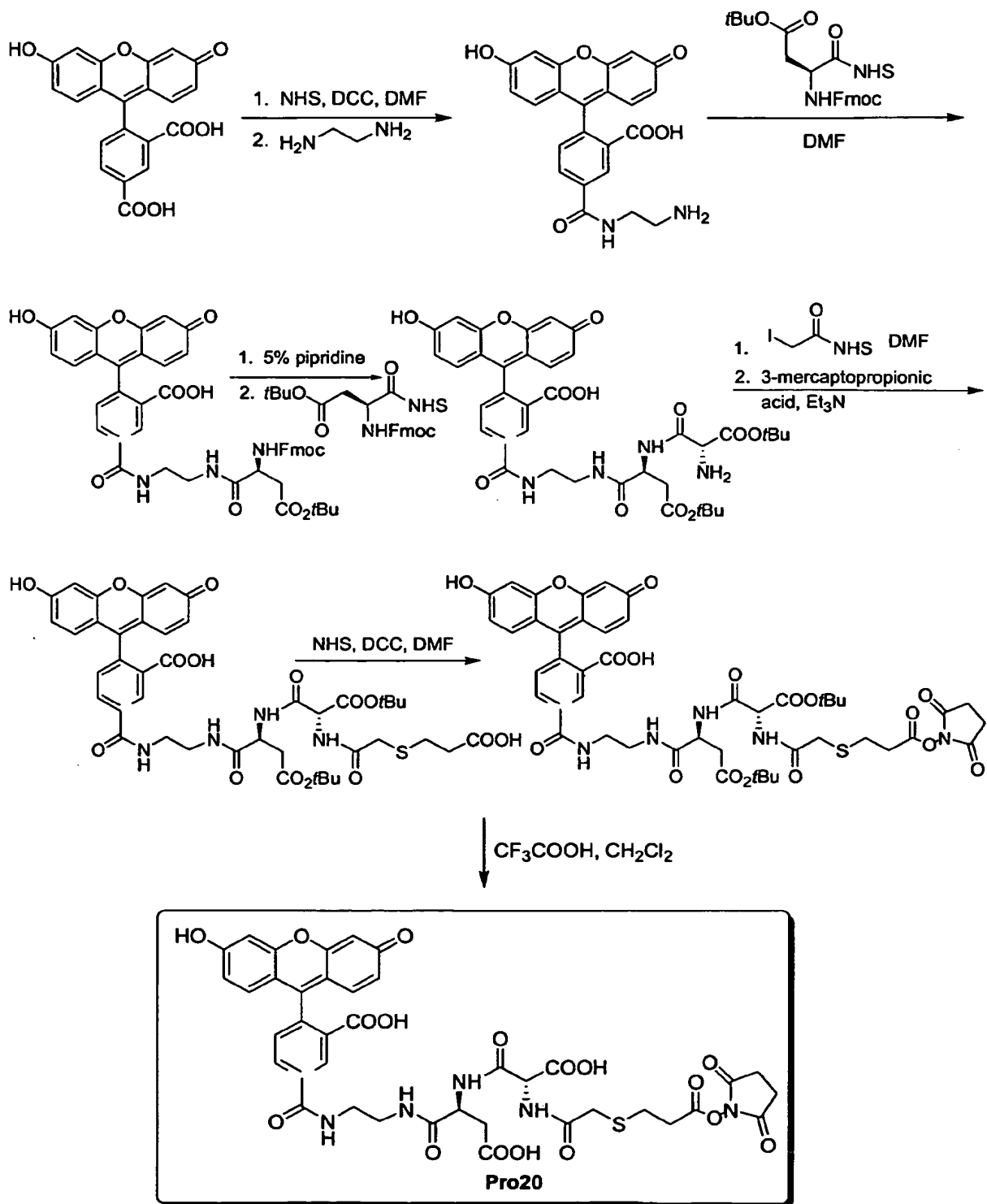
**Fig. 4J**

## Synthesis of Pro15



**Fig. 5A**

## Synthesis of Pro20



**Fig. 5B**

## Synthesis of Pro22

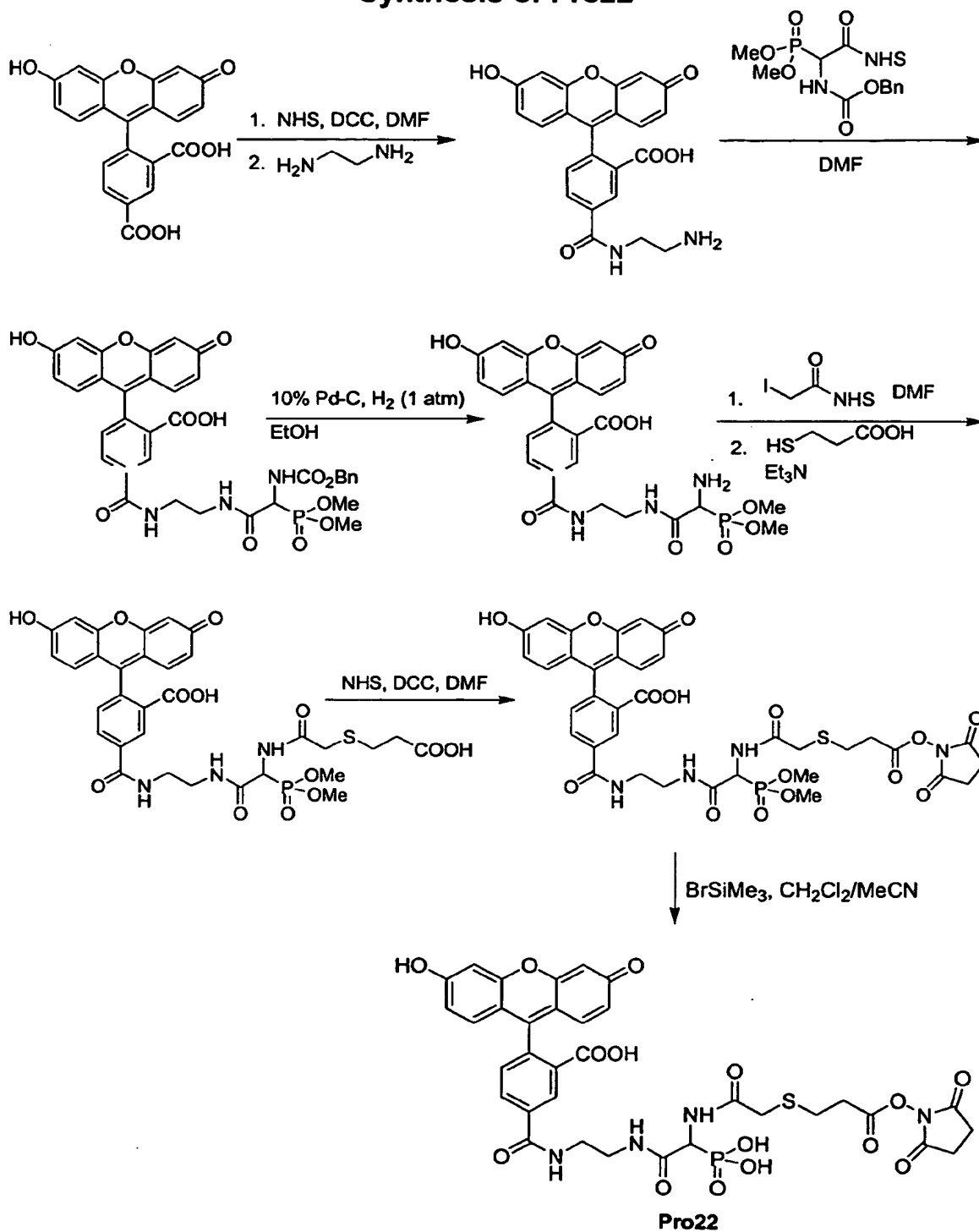


Fig. 5C

## Synthesis of Pro28

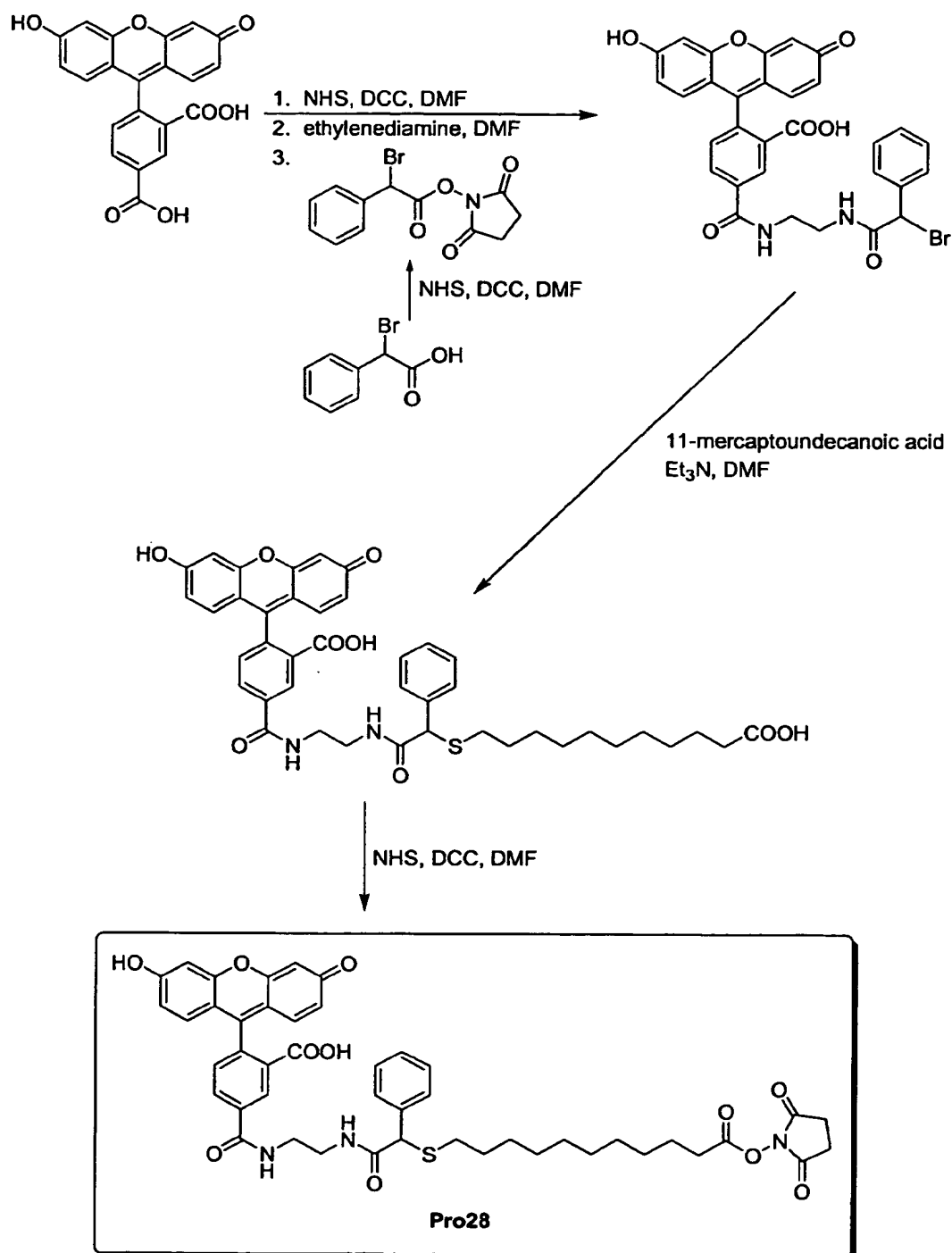
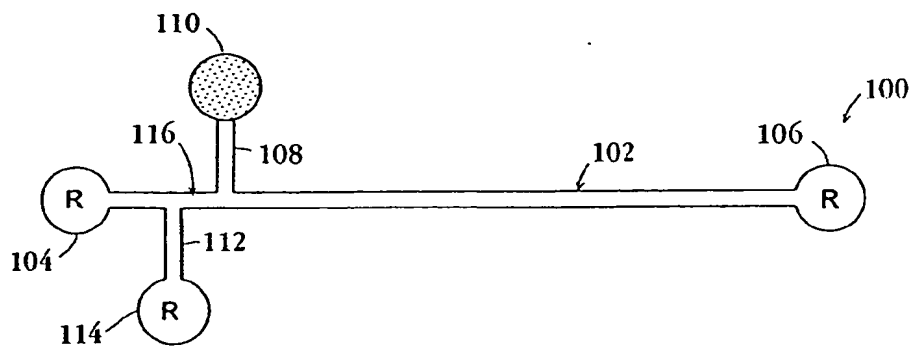
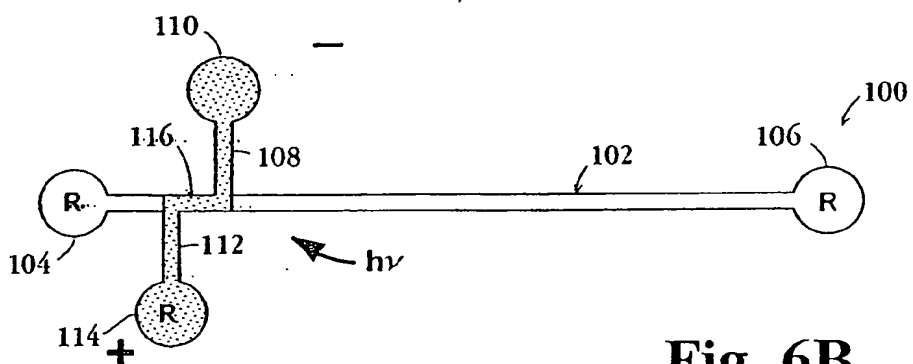


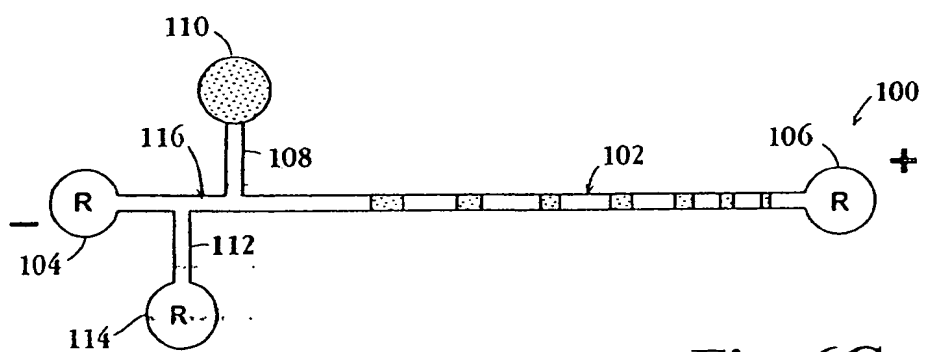
Fig. 5D



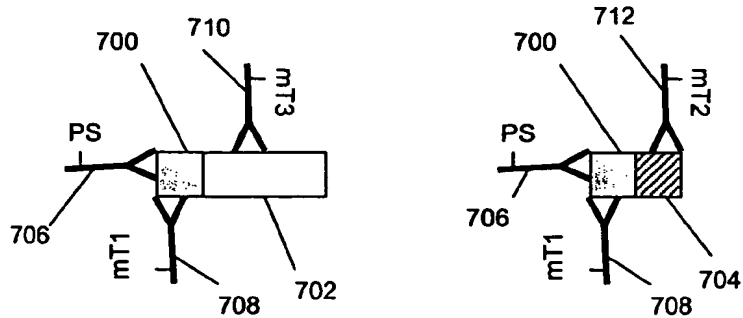
**Fig. 6A**



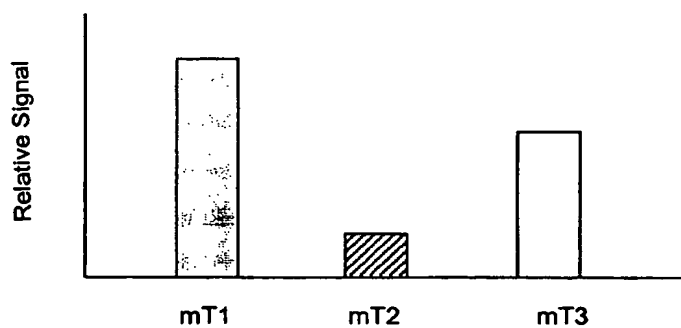
**Fig. 6B**



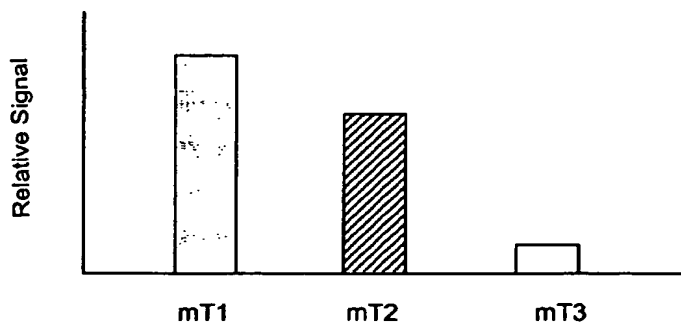
**Fig. 6C**



**Fig. 7A**



**Fig. 7B**



**Fig. 7C**

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